THE EFFECT OF MITOCHONDRIAL ULTRASTRUCTURE ON FUNCTION AND THERMAL TOLERANCE IN ANTARCTIC NOTOTHENIOID FISHES

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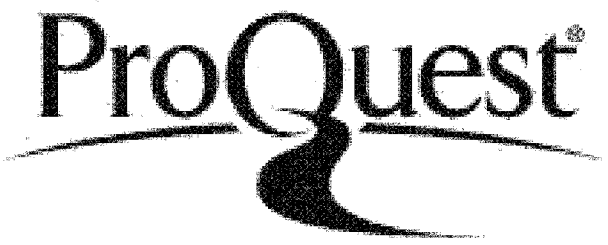

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ABSTRACT

The loss of hemoglobin in Antarctic icefishes is correlated with high mitochondrial volume densities and altered mitochondrial morphology in their oxidative muscle compared to red-blooded Antarctic notothenioid fish species. We hypothesized that differences in mitochondrial morphology between icefishes and red-blooded species might be correlated with differences in mitochondrial properties at their habitat temperature, near 0°C. We further hypothesized that differences in function might become more pronounced as temperature increases and might contribute to the lower thermal tolerance of icefishes compared to red-blooded species. Proton leak, rates of reactive oxygen species (ROS) production, membrane susceptibility to peroxidation and levels of antioxidants were measured in mitochondria isolated from hearts of the icefishes *Chaenocephalus aceratus* and *Chionodraco rastrospinosus,* and the red-blooded species *Gobionotothen gibberifrons* and *Notothenia coriiceps*. In addition, levels of oxidized proteins and lipids, and antioxidant levels were quantified in oxidative muscles of icefishes and red-blooded species exposed to their critical thermal maximum (CTmax) - an acute, short-term heat stress, and in animals exposed to 4°C for one week. Rates of ROS production increased as temperature increased in mitochondria isolated from both white- and red-blooded fishes. Yet, isolated mitochondria of icefishes are less protected against ROS. Antioxidant levels normalized to phospholipid content were lower in icefishes compared to red-blooded species, suggesting that icefishes might be more likely to experience oxidative stress as temperature increases. These findings were supported by measurements made in animals exposed to their CTmax. Levels of oxidized lipids increased in hearts of both icefishes, while levels of oxidized proteins increased only in *C. aceratus* in response to exposure to CTmax. In contrast, neither levels of oxidized lipids nor proteins increased in red-blooded fishes in response to exposure to CTmax. Similarly, levels of oxidized proteins did not increase in *C. rastrospinosus* or *N. coriiceps* in response to exposure to 4°C. Antioxidant levels tended to be lower in icefishes compared to red-blooded species and did not increase in any species in response to

exposure to CTmax or 4°C. In summary, our findings suggest that icefishes are more vulnerable to heat-induced oxidative stress compared to red-blooded fishes.

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GENERAL INTRODUCTION

The suborder Notothenioidei is the dominant group of fishes in the Southern Ocean, accounting for 35% of the fish species and approximately 91% of the fish biomass (Eastman, 1993a; 2005). Notothenioids include one of the most unique families of fishes, the Antarctic icefishes. Icefishes are the only adult vertebrates that lack the circulating oxygen-binding protein hemoglobin (Hb). The loss of Hb expression is due to a complete deletion of the ß-globin gene and a partial deletion of the α-globin gene (Ruud, 1954; Cocca et al., 1995). Oxygen is carried only in physical solution in the blood of icefishes, which reduces the oxygen-carrying capacity of their blood by 90% compared to red-blooded fishes (Ruud, 1954). The initial mutation leading to the loss of Hb might have been lethal, had it occurred in fishes living anywhere other than the Southern Ocean, which is cold and thermally stable. Temperatures in the Ross Sea are near -1.9°C all year around and fluctuate only slightly on a seasonal basis in the Western Antarctic Peninsula (WAP) region, ranging between -1.8°C and +2°C (Eastman, 1993b; Hofmann and Klinck, 1998; Jacobs et al., 2002). The solubility of oxygen is inversely correlated with temperature, allowing icefishes to transport 1.6-times more oxygen in their blood at 0°C than fish at 20°C (Clarke, 1983). In addition, icefishes have two- to three-times larger blood volumes compared to red-blooded fishes, further increasing the amount of oxygen transported in their blood (Holeton, 1976). The larger blood volume is pumped by hearts two- to four-times the size of red-blooded ones with a three- to five-times higher cardiac output, allowing icefishes to pump their blood at a high flow rate throughout the body and maintain a steep P_{O_2}-gradient between blood and tissue (Holeton, 1970; Hemmingsen et al., 1972; Hemmingsen, 1991). In addition, blood vessels of icefishes have two- to three-times larger diameters than vessels of red-blooded species, which reduces blood pressure, and high capillary densities increase the blood supply to some tissues of icefishes (Fitch et al., 1984; Hemmingsen, 1991; Wujcik et al., 2007). Together, cardiovascular adaptations and the cold environment of the Southern Ocean maintain the delivery of oxygen to tissues in icefishes.

In addition to the loss of Hb, six of the 16 species of the icefish also lack the intracellular oxygen-binding protein myoglobin (Mb) in their heart ventricles (Sidell et al., 1997; Vayda et al., 1997; Moylan and Sidell, 2000). Mb binds oxygen and acts as an intracellular oxygen reservoir, facilitating the diffusion of oxygen within tissues (reviewed in Sidell, 1998). In icefishes lacking Mb, the diffusion of oxygen within tissues is facilitated by a high volume density of mitochondria (O'Brien and Sidell, 2000; O'Brien et al., 2003; Urschel and O'Brien, 2008). Mitochondria account for 36.5% of the cell volume in cardiomyocytes in the Mb-less icefish *Chaenocephalus aceratus*, but displace only 18.2% and 15.9% of the cell volume in the red-blooded and red-hearted notothenioids *Notothenia coriiceps* and *Gobionotothen gibberifrons*, respectively (O'Brien and Sidell, 2000; Urschel and O'Brien, 2008). Mitochondrial volume densities are lower (20.1%) in cardiomyocytes of the Mb-expressing icefish *Chionodraco rastrospinosus* compared to *C. aceratus* (O'Brien and Sidell, 2000). High mitochondrial densities reduce the diffusion distance of oxygen between blood and mitochondria. In addition, they provide a membranous network within which oxygen diffuses more rapidly compared to the aqueous cytosol (reviewed in Sidell, 1998).

Mitochondrial volume density usually scales with aerobic capacity, but this is not the case in Antarctic icefishes. The maximal activities per gram of tissue of key metabolic enzymes such as citrate synthase (CS) and cytochrome c oxidase (CCO) are similar or lower in oxidative muscle of icefishes compared to red-blooded fishes, which is partially due to the altered ultrastructure of icefish mitochondria (O'Brien and Sidell, 2000; O'Brien et al., 2003). Mitochondria of icefishes are larger than mitochondria of red-blooded notothenioids and have less densely packed inner mitochondrial membranes (cristae) (O'Brien and Sidell, 2000). This suggests that the increase in mitochondrial size is achieved by incorporating newly-synthesized phospholipids into the outer but not inner mitochondrial membrane (O'Brien and Sidell, 2000; O'Brien and Mueller 2010). The combined high mitochondrial volume densities and low cristae surface densities yield nearly equivalent cristae densities per gram of tissue and therefore similar aerobic

capacities between muscles of icefishes and red-blooded notothenioids (O'Brien and Sidell, 2000).

Although Antarctic icefishes thrive in their cold environment, their future may be less promising. The WAP region is one of the fastest warming regions on Earth, with near-surface air temperatures increasing 10-times faster than the global average (IPCC, 2001; Vaughan et al., 2003; Turner et al., 2005). This warming trend might be a threat for Antarctic notothenioids, which have evolved in the thermally stable and cold environment of the Southern Ocean during the last 10-12 Million years, and thus are extremely well adapted to cold temperature (Eastman, 1993c). A high degree of stenothermy in animals is believed to be associated with a loss of thermal plasticity, and indeed, notothenioids have lost some, but not all, of their ability to respond to elevations in temperature. The most prominent example of the loss of thermal plasticity in Antarctic notothenioids is the loss of the heat shock response (HSR), which is found in organisms from all other taxa. Antarctic notothenioids express basal levels of heat shock proteins (Hsp) but are unable to up-regulate their expression in response to heat stress (Carpenter and Hofmann, 2000; Hofmann et al., 2000; Hofmann and Place, 2005). However, in response to heat stress, Antarctic notothenioids up-regulate some genes associated with transcriptional regulation, protein biosynthesis and carbohydrate and fatty acid metabolism (Buckley and Somero, 2009). In addition, warm acclimation of Antarctic notothenioids to 4°C significantly increased their survival time at 14°C and their critical thermal maximum (CTmax), which is the temperature at which animals lose equilibrium (Prodrabsky and Somero, 2006; Bilyk and Devries, 2011). The loss of some thermal plasticity might make Antarctic notothenioids vulnerable to increases in temperature.

Antarctic icefishes are more vulnerable to elevations in temperature compared to red-blooded notothenioids. Icefishes have a significantly lower CTmax compared to red-blooded notothenioids (Beers and Sidell, 2011). This is most likely due to the low oxygen-carrying capacity of their blood. The oxygen content of icefish blood will decline

as temperature increases because the solubility of oxygen is inversely proportional to temperature. At the same time, oxygen demand of tissues will increase because the rate of most enzymatic reactions increases approximately 2-times with each 10°C change in temperature, which is known as the Q_{10} effect (reviewed in Hochachka and Somero, 2002). Eventually, this will lead to a mismatch between oxygen supply and oxygen demand, causing hypoxia and impacting the animal's ability to survive.

The high mitochondrial volume densities and unusual mitochondrial ultrastructure of icefishes might also contribute to their lower thermal tolerance compared to red-blooded species. Studies in mammals have shown that changes in mitochondrial morphology are correlated with changes in mitochondrial properties, including rates of respiration, proton leak and rates of reactive oxygen species (ROS) production (Hackenbrock, 1966, Hackenbrock, 1968; Porter et al., 1996; John et al., 2005). Proton leak reduces the efficiency of ATP production because protons leak from intermembrane space into the mitochondrial matrix through the inner mitochondrial membrane or uncoupling proteins, bypassing ATP synthase (Brand, 2005). As a result, more oxygen is needed to produce equivalent amounts of ATP. Proton leak increases as temperature increases, which might magnify the mismatch between oxygen supply and oxygen demand as temperature increases (Chamberlin, 2004). Furthermore, high rates of ROS production can oxidatively damage macromolecules (reviewed in Ott et al., 2007). Similar to proton leak, rates of ROS production increase with temperature in ectotherms (Abele et al., 2002; Heise et al., 2003; Keller et al., 2004). Differences in rates of ROS production between mitochondria of icefishes and red-blooded species might make icefishes more likely to experience higher ROS challenges as temperatures increases. In addition, high mitochondrial volume densities in oxidative muscles of icefishes can further facilitate the production of ROS via the lipid peroxidation cycle (reviewed in Girotti, 1998).

High rates of ROS production are not problematic when counterbalanced by antioxidants. The first antioxidant in the defense against ROS produced by mitochondria is superoxide dismutase (SOD), which converts superoxide anions into hydrogen peroxide. Hydrogen peroxide in turn is reduced to water by several enzymes, including catalase, thioredoxin and glutathione peroxidase. In addition to antioxidant enzymes, several low molecular weight antioxidants such as glutathione, vitamin E and vitamin C protect biological macromolecules from oxidative damage (reviewed in Chaudiere and Ferrari-Iliou, 1999). Levels of antioxidants are significantly lower in icefishes than red-blooded notothenioids, suggesting they may be less well protected against ROS compared to red-blooded fishes (Witas et al., 1984; Cassini et al., 1993; Dunlap et al., 2002). Maximal activity of SOD is up to 6.5-times lower in icefishes compared to red-blooded notothenioids (Witas et al., 1984; Cassini et al., 1993). Similarly, maximal activity of catalase is up to 4.0-times lower in icefishes compared to red-blooded fishes (Cassini et al., 1993). The same trend is found in levels of non-enzymatic antioxidants: vitamin E and its derivates are up to 13.0-times lower in membranes of icefishes compared to red-blooded species (Dunlap et al., 2002). In addition, it is not clear yet whether Antarctic notothenioids are able to up-regulate their antioxidant defenses to combat temperature-dependent increases in ROS. A previous study using cDNA microarrays of the red-blooded notothenioid *Trematomus bernacchii* suggests that Antarctic fishes might experience oxidative stress in response to a 4°C heat shock because genes involved in protein degradation, protein biosynthesis and protein folding, as well as DNA replication and repair were up-regulated during heat stress and during the first four hours of recovery at -1.8°C (Buckley and Somero, 2009). However, antioxidants were not up-regulated during heat shock or recovery, suggesting that the ability to up-regulate antioxidants in response to elevations in temperature might have been lost in Antarctic notothenioids. Alternatively, changes in the expression of antioxidants may not have been detected because only 20-27% of genes could be identified in this study (Buckley and Somero, 2009).

The overall aim of this study was to determine whether differences in mitochondrial ultrastructure between icefishes and red-blooded notothenioids affect mitochondrial properties and whether these differences contribute to the reduced thermal tolerance of icefishes compared to red-blooded species. This aim was addressed in the following three research objectives:

1) *Determine whether properties of isolated mitochondria differ between icefishes and red-blooded notothenioids close to habitat temperature (2°C) and at 10°C.*

State III respiration rates, proton leak and rates of ROS production were quantified in mitochondria isolated from the heart ventricle of the icefishes *C. aceratus* (-Mb) and *C. rastrospinosus* (+Mb) and the two red-blooded species *G. gibberifrons* and *N. coriiceps* at 2°C and 10°C. Furthermore, maximal activity of SOD was quantified in mitochondria isolated from *C. aceratus* and *N. coriiceps*, while membrane susceptibility to lipid peroxidation and total antioxidant capacity were quantified in mitochondria isolated from *C. aceratus* and *N. coriiceps* by our collaborators at Ohio University, Athens.

2) *Determine whether red- and white-blooded Antarctic fishes experience oxidative stress* in vivo *in response to acute heat stress, and if these animals can up-regulate their antioxidant defenses.*

Levels of oxidized proteins, and transcript levels as well as maximal activity of the antioxidants SOD and catalase were quantified in heart ventricle and pectoral muscle of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps*, which were exposed to 0°C or their CTmax. In addition, levels of oxidized lipids were quantified in oxidative muscles of all four species by our collaborators at Ohio University, Athens.

3) *Determine whether red- and white-blooded Antarctic fishes experience oxidative stress* in vivo *in response to a prolonged increase in temperature, and if these animals can defend themselves against oxidative stress by up-regulating their antioxidant defense.*

Levels of oxidized proteins, and transcript levels as well as maximal activity of the SOD and catalase were quantified in heart ventricle and pectoral muscle of *C. rastrospinosus* and *N. coriiceps* maintained at 0°C or 4°C for one week.

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CHAPTER 1: INTER-RELATIONSHIP BETWEEN MITOCHONDRIAL FUNCTION AND SUSCEPTIBILITY TO OXIDATIVE STRESS IN RED- AND WHITE-BLOODED ANTARCTIC NOTOTHENIOID FISHES[1]

[1] Mueller, I. A., Grim, J. M., Beers, J. M., Crockett, E. L. and O'Brien, K. M. 2011. Inter-relationship between mitochondrial function and susceptibility to oxidative stress in red- and white-blooded Antarctic notothenioid fishes. *Journal of Experimental Biology* 214(22): 3732-3741

1.1 SUMMARY

It is unknown whether Antarctic fishes can defend themselves against oxidative stress induced by elevations in temperature. We hypothesized that Antarctic icefishes, lacking the oxygen-binding protein hemoglobin, might be more vulnerable to temperature-induced oxidative stress compared with red-blooded notothenioids because of differences in their mitochondrial properties. Mitochondria from icefishes have higher densities of phospholipids per milligram of mitochondrial protein compared with red-blooded species, and these phospholipids are rich in polyunsaturated fatty acids (PUFAs), which can promote the formation of reactive oxygen species (ROS). Additionally, previous studies have shown that multiple tissues in icefishes have lower levels of antioxidants compared with red-blooded species. We quantified several mitochondrial properties, including proton leak, rates of ROS production, membrane composition and susceptibility to lipid peroxidation (LPO), the activity of superoxide dismutase (SOD) and total antioxidant power (TAOP) in mitochondria isolated from hearts of icefishes and red-blooded notothenioids. Mitochondria from icefishes were more tightly coupled than those of red-blooded fishes at both 2°C and 10°C, which increased the production of ROS when the electron transport chain was disrupted. The activity of SOD and TAOP per milligram of mitochondrial protein was equivalent between icefishes and red-blooded species, but TAOP normalized to mitochondrial phospholipid content was significantly lower in icefishes compared with red-blooded fishes. Additionally, membrane susceptibility to peroxidation was only detectable in icefishes at 1°C and not in red-blooded species. Together, our results suggest that the high density of mitochondrial phospholipids in hearts of icefishes may make them particularly vulnerable to oxidative stress as temperatures rise.

1.2 INTRODUCTION

Notothenioid fishes have inhabited the thermally stable and cold environment of the Southern Ocean for ~10–12 million years (Eastman, 1993). Water temperatures south of the Antarctic Polar Front range between –1.9°C and 3°C, and fluctuate minimally on a seasonal basis (Littlepage, 1965; Eastman, 1993; Hunt et al., 2003). Notothenioids possess an array of adaptations that make them extraordinarily well-suited for life in the cold, including antifreeze proteins and cold-stable microtubules (Cheng and Detrich, 2007). The ability of notothenioids to withstand elevations in temperature is less clear. The upper incipient lethal temperature limit of three species of notothenioids was determined to be only between 5°C and 7°C (Somero and DeVries, 1967). However, more recent studies have revealed that notothenioids have a limited capacity to acclimate to warmer temperatures, as evidenced by changes in thermal tolerance, cardiac function and antifreeze levels in response to an increase in temperature (Jin and DeVries, 2006; Podrabsky and Somero, 2006; Franklin et al., 2007; Bilyk and Devries, 2011). The ability of Antarctic fishes to withstand warming of the Southern Ocean, which is occurring very rapidly in the Western Antarctic Peninsula (WAP) region (Vaughan et al., 2003), will be dependent, at least in part, on their ability to maintain mitochondrial function over a range of temperatures.

Channichthyid icefishes are among the most notable families of Antarctic notothenioids, distinguished by their lack of expression of the oxygen-binding protein hemoglobin (Hb) (Ruud, 1954). Six of the 16 species of icefishes also lack the intracellular oxygen-binding protein myoglobin (Mb) in their heart ventricle, which stores and facilitates the diffusion of oxygen within oxidative muscle (Sidell et al., 1997; Moylan and Sidell, 2000). The loss of Hb expression reduces the blood oxygen-carrying capacity of icefishes to one-tenth that of red-blooded species (Ruud, 1954). Icefishes have likely survived without Hb and Mb because of their cold, well-oxygenated environment, together with several modifications in their cardiovascular system, which

enhance oxygen delivery (reviewed by Sidell and O'Brien, 2006). However, as global temperatures rise, the loss of Hb may become disadvantageous. The solubility of oxygen in blood plasma is inversely correlated with temperature, so that as temperature increases, blood oxygen-carrying capacity of icefishes is more likely to decline compared with Hb-expressing fish. Current empirical evidence supports this conjecture. The critical thermal maximum, defined as the temperature at which fish lose the ability to right themselves, is positively correlated with hematocrit and is 1.5–3.0°C lower in icefishes compared with red-blooded notothenioids (Beers and Sidell, 2011).

Differences in the properties of mitochondria between red- and white-blooded notothenioids may impact the balance between oxygen supply and demand and could therefore influence thermal tolerance. Mitochondrial function is inextricably linked to structure, which is strikingly different between red- and white-blooded notothenioids. Mitochondria from icefishes are 1.1-times to 1.4-times larger with more loosely packed inner membranes (cristae) compared with mitochondria from red-blooded notothenioids (O'Brien and Sidell, 2000). Alterations in mitochondrial morphology can affect several aspects of mitochondrial function, including proton leak (Porter et al., 1996), which occurs when protons leak across the inner mitochondrial membrane from the intermembrane space into the mitochondrial matrix, bypassing the ATP synthase enzyme (reviewed by Brand, 2005). Proton leak is affected by the composition of mitochondrial membranes (Brookes et al., 1998) and is positively correlated with the density of inner mitochondrial membranes (Porter et al., 1996), the activity of uncoupling proteins (UCPs) (Brand et al., 2004) and the adenosine nucleotide translocase (ANT) enzyme (Brand et al., 2005) and, notably, temperature (Chamberlin, 2004; Jastroch et al., 2007). As temperature and proton leak increase, more oxygen will be required by the respiratory chain to generate a proton gradient and maintain ATP production. This may be particularly problematic for icefishes with a reduced blood oxygen-carrying capacity compared with red-blooded species.

Compared with red-blooded species, icefishes may also be less thermally tolerant because properties of their mitochondria could place them at a greater risk for oxidative damage as temperature increases. Oxidative stress occurs when the rate of production of reactive oxygen species (ROS) exceeds antioxidant defenses, resulting in oxidatively damaged macromolecules (reviewed by Halliwell, 2011). The majority of ROS are produced by complexes I and III of the mitochondrial respiratory chain when electrons leak from redox centers and react with oxygen, forming superoxide (reviewed by Turrens, 2003). Rates of ROS production are influenced by rates of cellular respiration, the degree of mitochondrial coupling and the presence of polyunsaturated fatty acids (PUFAs). Studies of mitochondria isolated from ectotherms have shown that as temperature increases, oxygen consumption and the production of ROS increases (Abele et al., 2002; Heise et al., 2003; Keller et al., 2004). This effect may be magnified *in vivo* in the presence of PUFAs, which propagate the formation of ROS via the lipid peroxidation (LPO) cycle (reviewed by Girotti, 1998; Crockett, 2008). Mitochondria from icefishes may be particularly vulnerable to oxidative damage at elevated temperatures because they are more lipid-rich compared with those from red-blooded fishes (O'Brien and Mueller, 2010). Levels of two of the major mitochondrial phospholipids, phosphatidylethanolamine (PE) and phosphatidylcholine (PC), are 1.3-times to 1.4-times higher per milligram of mitochondrial protein in mitochondria from the icefish *Chaenocephalus aceratus* compared with those from the red-blooded species *Notothenia coriiceps*. Potentially compounding the problem, oxidative muscles of icefishes have mitochondrial densities up to 2.3-times higher than red-blooded species (reviewed by O'Brien and Mueller, 2010). The high density of lipid-rich mitochondria in oxidative muscles of icefishes enhances oxygen delivery in the absence of Hb and Mb (reviewed by O'Brien, 2011). Although beneficial at the current cold temperature of the Southern Ocean, mitochondria and tissues rich in polyunsaturated phospholipids may be a liability as temperature increases because they promote the formation of ROS (Cosgrove et al., 1987). Moreover, previous studies suggest that icefishes have a lower capacity to detoxify ROS compared with red-blooded species (Cassini et al., 1993; Witas

et al., 1984). The activity of superoxide dismutase (SOD) is 6-times lower and the activity of catalase enzyme is 4-times lower in hearts of the icefish *Chionodraco hamatus* compared with the red-blooded species *Trematomus bernacchii* (Cassini et al., 1993). Low levels of antioxidants coupled with high levels of PUFAs may make icefishes especially vulnerable to oxidative stress as the Southern Ocean warms. The WAP, where animals for this study were obtained, is one of the fastest warming regions on Earth, with sea surface temperatures increasing more than 1°C since 1951 (Meredith and King, 2005; Schofield et al., 2010). The top 100 m of continental shelf waters in the WAP vary annually by ~3°C whereas deeper waters, originating as circumpolar deep water (CDW), are more thermally stable and range between 1°C and 1.5°C (Barnes and Peck, 2008; Clarke et al., 2009).

We hypothesized that differences in mitochondrial structure and function between red- and white-blooded notothenioids might contribute to differences in thermal tolerance. To test this hypothesis, we measured proton leak, mitochondrial membrane composition, rates of ROS production, susceptibility of mitochondrial membranes to LPO, the activity of SOD and total antioxidant power (TAOP) in mitochondria isolated from hearts of red- and white-blooded notothenioids. Mitochondria from heart ventricles were used because the heart is highly aerobic and contains a high density of mitochondria. Additionally, we have developed techniques for isolating intact, well-coupled mitochondria from heart tissue (O'Brien and Sidell, 2000; Urschel and O'Brien, 2008). Most measurements were made at temperatures close to physiological temperature (1–2°C) and at the elevated temperature of 10°C to determine if mitochondrial susceptibility to oxidative stress induced by warming differs between red- and white-blooded notothenioid fishes.

1.3 MATERIAL AND METHODS

1.3.1 Tissue collection

Chaenocephalus aceratus (–Hb/–Mb) (Lönnberg), *Chionodraco rastrospinosus* (–Hb/+Mb) (Dewitt and Hureau), *Notothenia coriiceps* (+Hb/+Mb) (Richardson) and *Gobionotothen gibberifrons* (+Hb/+Mb) (Lönnberg) were captured in Dallmann Bay (64°S, 62°W) during the austral autumn of 2009 using an otter trawl deployed from the ARSV *Laurence M. Gould*. *N. coriiceps* were also captured using baited traps. Fish were maintained in circulating seawater tanks onboard the *Laurence M. Gould* and then transferred to circulating seawater tanks at the U.S. Antarctic Research Station, Palmer Station, where they were held at 0 ± 1°C. Animals were killed by a sharp blow to the head followed by transecting the spinal cord. Heart ventricles were quickly excised and frozen in liquid nitrogen unless experiments required fresh tissue. Frozen tissues were stored at –80°C. All procedures were approved by the University of Alaska Fairbanks Institutional Animal Care Committee (134774-2).

1.3.2 Mitochondrial isolation

Heart ventricles were excised as described above, placed in ice-cold Ringer solution (240 mmol l^{-1} NaCl, 2.5 mmol l^{-1} $MgCl_2$, 5.0 mmol l^{-1} KCl, 2.5 mmol l^{-1} $NaHCO_3$, 5.0 mmol l^{-1} NaH_2PO_4, pH 8.0), and allowed to contract several times to clear blood from the ventricular lumen. Two ventricles from *C. rastrospinosus* or *N. coriiceps*, and 5–6 ventricles from *G. gibberifrons* were pooled for each mitochondrial preparation. Ventricles were homogenized in 8 volumes of ice-cold isolation buffer (0.1 mol l^{-1} sucrose, 140 mmol l^{-1} KCl, 10 mmol l^{-1} EDTA, 5 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} HEPES, 0.5% fatty acid-free bovine serum albumin (BSA), pH 7.3 at 4°C) using a Tekmar Tissuemizer (TeledyneTekmar, Cincinnati, OH, USA) set at low speed for 3 sec. Homogenization was completed by hand using a 40 ml Tenbroeck ground glass

homogenizer (Wheaton, Millville, NJ, USA). Mitochondria were separated by differential centrifugation as described previously by Urschel and O'Brien (Urschel and O'Brien, 2009). Mitochondrial pellets were resuspended in assay buffer (173 mmol l^{-1} sucrose, 135 mmol l^{-1} KCl, 5 mmol l^{-1} KH_2PO_4, 20 mmol l^{-1} HEPES, 0.5% fatty acid-free BSA, pH 7.3 at 4°C). BSA was omitted from all buffers for isolating mitochondria used to quantify rates of ROS production because BSA interferes with detecting resorufin. Despite the lack of BSA, the respiratory control ratio (RCR) was > 5 in mitochondria, indicating well-coupled mitochondria. For analyzing mitochondrial membrane composition, measuring the susceptibility of mitochondrial membranes to peroxidation, and the activity of SOD and TAOP, heart ventricles were homogenized in 8 volumes of isolation buffer without sucrose, and BSA was omitted from wash buffer. Mitochondria were resuspended in 10 mmol l^{-1} Tris buffer, pH 7.3 at 4°C, frozen in liquid nitrogen and stored at –80°C for these measurements. Protein concentration of all mitochondrial preparations was determined using the bicinchoninic acid (BCA) assay (Smith et al., 1985).

The RCR was measured at 2°C and 10°C to verify the quality of mitochondria prior to measuring proton leak, and only mitochondria with an RCR > 5 were used. Mitochondria were resuspended in oxygenated assay buffer (173 mmol l^{-1} sucrose, 135 mmol l^{-1} KCl, 5 mmol l^{-1} KH_2PO_4, 20 mmol l^{-1} HEPES, 0.5% fatty acid-free BSA, pH 7.3 at each assay temperature) as described previously by Urschel and O'Brien (Urschel and O'Brien, 2009). Mitochondrial state III respiration rates were measured using a Strathkelvin oxygen electrode (Strathkelvin Instruments, North Lanarkshire, Scotland, UK) with 5 mmol l^{-1} pyruvate, 1 mmol l^{-1} malate and 0.6 mmol l^{-1} ADP added as substrates. State III respiration rates were measured for 3–8 min following the addition of ADP, and oxygen consumption was measured for an additional 3 min following ADP depletion to determine state IV respiration rates.

1.3.3 Proton leak

Proton leak was measured in isolated mitochondria at 2°C and 10°C. Rates of state II respiration were quantified using a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, UK), and mitochondrial membrane potential was measured using a triphenylmethylphosphonium ($TPMP^+$)-sensitive electrode as described by Brand (Brand, 1995). Assay buffer (173 mmol l^{-1} sucrose, 135 mmol l^{-1} KCl, 5 mmol l^{-1} KH_2PO_4, 20 mmol l^{-1} HEPES, 0.5% fatty acid-free BSA, pH 7.3 at each assay temperature) was oxygenated for 5 min with constant stirring. Mitochondria were then added, along with 5 mmol l^{-1} rotenone, 1 mg ml^{-1} oligomycin and 80 ng ml^{-1} nigericin. A $TPMP^+$ standard curve was generated by adding five aliquots of 0.5 mmol l^{-1} $TPMP^+$. State II respiration rates were measured for 3–12 min following the addition of 4 mmol l^{-1} succinate. State II respiration was then gradually inhibited by adding increasing concentrations of malonate every 3 min, beginning with 0.2 mmol l^{-1} and increasing to a final concentration of 3.2 mmol l^{-1}. Mitochondria were then uncoupled by adding 0.29 mmol l^{-1} carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) to correct for drift of the $TPMP^+$-sensitive electrode. All measurements were made in duplicate in 4–5 mitochondrial preparations per species. Rates of state II respiration were plotted against mitochondrial membrane potential to obtain proton leak curves. Mitochondrial membrane potential was calculated from the response of the $TPMP^+$-sensitive electrode according to the Nernst equation.

Non-specific binding of $TPMP^+$ to the membrane was measured at 2°C and 10°C in two mitochondrial preparations from *C. aceratus*, *C. rastrospinosus* and *N. coriiceps* as described by Lotscher et al. (Lotscher et al., 1980). Non-specific binding of $TPMP^+$ to mitochondrial membranes of *G. gibberifrons* was assumed to be equivalent to that of *N. coriiceps*. Mitochondrial matrix volume was measured in *C. aceratus* and *N. coriiceps* at 1°C as described by Brand (Brand, 1995). Previous studies have shown that mitochondrial matrix volume does not change with temperature (Chamberlin, 2004). Measurements were made in duplicate in 4–6 mitochondrial preparations per species.

Mitochondrial matrix volume of *C. rastrospinosus* was determined by plotting mitochondrial surface-to-volume ratio against mitochondrial matrix volume for *N. coriiceps* and *C. aceratus*, and using previous stereological measurements of mitochondrial surface-to-volume ratio for *C. rastrospinosus* (O'Brien and Sidell, 2000). Mitochondrial matrix volume of *G. gibberifrons* was assumed to be the same as the matrix volume of *N. coriiceps* because the mitochondrial surface-to-volume ratio is equivalent between these two species (O'Brien and Sidell, 2000; Urschel and O'Brien, 2008). Mitochondrial matrix volume and corrections factors for non-specific binding of $TPMP^+$ are shown in Appendix 1.1.

1.3.4 Rates of ROS production

Rates of ROS production were measured in mitochondria isolated from *C. aceratus* and *N. coriiceps* at 2°C and 10°C by monitoring the rate of formation of resorufin at 572 nm using a Perkin-Elmer Lambda 40 spectrophotometer (Perkin-Elmer Corp., Waltham, MA, USA) as described by Chen et al. (Chen et al., 2003). Mitochondria were incubated in assay buffer (173 mmol l^{-1} sucrose, 135 mmol l^{-1} KCl, 5 mmol l^{-1} KH_2PO_4, 20 mmol l^{-1} HEPES, pH 7.3 at each assay temperature) containing 50 mmol l^{-1} Amplex Red, 0.2 U ml^{-1} horseradish peroxidase, 30 U ml^{-1} SOD and 5 mmol l^{-1} succinate for 60 min. 5 mmol l^{-1} rotenone or 10 mmol l^{-1} antimycin A were added to inhibit complex I or III, respectively. 1 mmol l^{-1} malate and 2.5 mmol l^{-1} pyruvate were used as substrates in place of succinate when the respiratory chain was inhibited with rotenone. Hydrogen peroxide was serially diluted (0-1 mmol) to create a standard curve for calculating rates of ROS production. All measurements were done in duplicate in 10 mitochondrial preparations per species.

1.3.5 Susceptibility to LPO

The susceptibility of mitochondrial membranes to LPO was quantified using the fluorometric probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY 581/591) as described previously (Drummen et al., 2002; Grim et al., 2010). LPO was induced with hydroxyl radicals generated by the Fenton reaction between Cu^{2+} (as copper sulfate) and cumene hydroperoxide (CumOOH). Membranes were diluted to 0.05 mg ml^{-1} protein using 20 mmol l^{-1} Chelex®-Tris (pH 7.4). A working BODIPY stock (1 mmol l^{-1} in 100% ethanol) was diluted to 10 mmol l^{-1} with 20 mmol l^{-1} Chelex®-Tris (pH 7.4). This probe solution was further diluted to a final concentration of 148 nmol l^{-1} with the 0.05 mg ml^{-1} protein solution (final protein concentration of 0.05 mg ml^{-1}). The probe was dispersed within the membrane by stirring slowly in the dark at 4°C for 60 min. Subsequently, LPO was induced in duplicate cuvettes containing 2.5 ml of membrane/probe solution by adding 38 μl of 822 mmol l^{-1} $CuSO_4$ and 82 μl of 20 mmol l^{-1} Chelex®-Tris (pH 7.4), followed 5 min later by the addition of 38 μl of 3.3 mmol l^{-1} CumOOH and 82 μl of 20 mmol l^{-1} Chelex®-Tris (pH 7.4) (total volume of 2.74 ml and final inductant concentrations of 11.5 mmol l^{-1} and 46 mmol l^{-1}, respectively). The inductant load was titrated at 10°C until a small linear slope was observed (3.5–6 min), and subsequent increases in inductant load did not substantially increase linear rates of LPO. This titration step ensured that the same inducant challenge would be sufficient to produce a measurable rate of LPO when assayed at 1°C. Fluorescence decay was followed at both 10°C and 1°C at excitation/emission wavelengths of 568 nm/590 nm, using an LS-50B spectrofluorometer (Perkin-Elmer Corp., Waltham, MA, USA). Linear portions of the decay slope represented rates of LPO, and an extinction coefficient of 139,444 l mol^{-1} cm^{-1} was used in all calculations (Drummen et al., 2004). Rates of LPO were normalized to protein content and phospholipid content measured according to Rouser et al. (Rouser et al., 1970).

1.3.6 Lipid extraction

Lipids were extracted and analyzed from mitochondria of *C. aceratus* and *N. coriiceps* as described by Yang et al. (Yang et al., 2009). Briefly, mitochondria were homogenized in 3 volumes (v/w of mitochondria) of chloroform/methanol (1:2). The extraction mixture was further diluted with 1 volume (v/w of mitochondria) of chloroform and 1 volume (v/w of mitochondria) of distilled water. The extraction mixture was centrifuged (10 min, 0.5 ***g***) to separate the chloroform and aqueous methanol, and then chloroform was carefully removed. 2 ml of chloroform/methanol (1:1) were added to the remaining aqueous phase and the chloroform separated and removed as described above. The chloroform was dried under nitrogen stream, resuspended in 4 ml of chloroform/methanol (1:1), re-extracted in 1.8 ml of 20 mmol l^{-1} aqueous LiCl and dried as described above. The lipid extracts were resuspended in chloroform/methanol (1:1) at a final volume of 500 mg ml^{-1} protein and further diluted with chloroform/methanol/isopropanol (1:2:4) to a final concentration of less than 50 pmol ml^{-1} of total phospholipid prior to mass spectroscopy analysis. Lipids were analyzed using a TSQ Quantum Ultra Plus triple-stage quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA).

The equation used for calculating unsaturation index (UI) was modified from Hulbert et al. (Hulbert et al., 2007) by Grim et al. (Grim et al., 2010) to account for the presence of four acyl chains in cardiolipin, resulting in a maximum of 24 double bonds per molecule of cardiolipin (6 double bonds within each of its 4 acyl chains):

$$\mathrm{UI} = \sum_{n=24}^{n=0} n \text{ x mol\% of fatty acids containing } n \text{ double bonds} \qquad \text{(Eq. 1.1)}$$

1.3.7 SOD activity (EC 1.15.1.1)

The activity of SOD was measured in isolated mitochondria using a modified method (Crapo et al., 1978) of the xanthine oxidase (XO)/cytochrome *c* protocol originally described by McCord and Fridovich (McCord and Fridovich, 1969). This method is based on the ability of SOD to inhibit the reduction of cytochrome *c* by superoxide. The reaction mixture contained 50 mmol l^{-1} potassium phosphate (pH 7.8), 0.1 mmol l^{-1} EDTA, 0.05 mmol l^{-1} xanthine, 0.01 mmol l^{-1} acetylated cytochrome *c* (equine heart), 0.01 mmol l^{-1} KCN and ~0.07 U XO. The final concentration of XO was determined empirically so that the reduction of cytochrome *c*, detected at 550 nm, occurred at a rate of 0.02 OD min^{-1}. All assays were performed in duplicate at 5.0 ± 0.5°C using a Perkin-Elmer Lambda 40 spectrophotometer (Perkin-Elmer Corp., Waltham, MA, USA). Temperature was regulated using a refrigerated, circulating water bath connected to the spectrophotometer. One unit of activity is defined as the amount of SOD needed to inhibit the reduction of cytochrome *c* by 50%.

1.3.8 TAOP

TAOP was measured in isolated mitochondria using a TAOP kit (Oxford Bioresearch, Manchester Hills, MI, USA). This kit uses a colorimetric endpoint assay that determines the reduction of copper coupled to bathocuprione. Samples were diluted 80-times, which included an initial 2-times dilution in PBS followed by a 40-times dilution in the buffer provided by the manufacturer, as per the manufacturer's instructions. Standard curves using uric acid (0–2 mmol l^{-1}) were measured with each set of samples. Both standards and samples were run in duplicate. TAOP is expressed in uric acid equivalents mg of $protein^{-1}$ or mmol of $phospholipid^{-1}$.

1.3.9 Statistical analyses

Significant differences in the rates of state II respiration measured at a common membrane potential, rates of ROS production within a species at different temperatures and between species at a common temperature, mean LPO susceptibility (normalized to protein content and phospholipid content), metrics of phospholipid content (abundance of individual phospholipid species and phospholipid classes), membrane unsaturation, activity of SOD and TAOP were compared using a Student's *t*-test. All statistical analyses were done using the software JMP (JMP5 or JMP7; SAS, Cary, NC, USA). Data were log transformed as necessary to maintain assumptions of normality. Data not meeting assumptions of normality or homogeneity of variance were compared with Wilcoxon rank sums or Welch ANOVA, respectively. Significance was set at $P < 0.05$. Unless otherwise noted, data are presented as means ± standard error of the mean (s.e.m.).

1.4 RESULTS

1.4.1 Proton leak

Proton leak was greater in mitochondria isolated from red-blooded notothenioids compared with icefishes at both 2°C and 10°C (Fig. 1.1). To compare proton leak among species or treatments, state II respiration rates are compared at the highest common membrane potential (e.g. Brookes et al., 1998; Jastroch et al., 2007). State II respiration rates were 16.1-times higher in mitochondria from *N. coriiceps* and 17.2-times higher in mitochondria from *G. gibberifrons* compared with *C. aceratus* at a common membrane potential of 190 mV at 2°C (Table 1.1). State II respiration rates were 4.4-times higher in mitochondria from *C. rastrospinosus* compared with *C. aceratus* at a common membrane potential of 190 mV at 2°C (Table 1.1). State II respiration rates were not significantly

different between mitochondria of *N. coriiceps* and *G. gibberifrons* at 190 mV and 2°C ($P > 0.05$, Table 1.1). Proton leak was higher in mitochondria of all four species at 10°C compared with 2°C, but remained lower in icefishes compared with red-blooded notothenioids (Fig. 1.1 A, B). Proton leak was lowest in mitochondria of *C. aceratus* at a common membrane potential of 147 mV at 10°C compared with all other species (Fig. 1.1 B, Table 1.1). State II respiration rates were 3.7-times higher in mitochondria of *C. rastrospinosus*, 9.2-times higher in *N. coriiceps* and 12.2-times higher in *G. gibberifrons* compared with *C. aceratus*, but were not significantly different between mitochondria of *N. coriiceps* and *G. gibberifrons* at 147 mV and 10°C ($P > 0.05$, Table 1.1). Together, these findings indicate that proton leak is lowest in mitochondria of *C. aceratus*, intermediate in *C. rastrospinosus* and highest in *N. coriiceps* and *G. gibberifrons* at 10°C (Fig. 1.1 B).

1.4.2 Rates of ROS production

Rates of ROS production were not significantly different between mitochondria from *C. aceratus* and *N. coriiceps* at 2°C or 10°C ($P > 0.05$, Fig. 1.2). In both species, rates of ROS production were significantly higher at 10°C compared with 2°C ($P < 0.05$, Fig. 1.2). However, rates of ROS production were significantly higher in mitochondria of *C. aceratus* at both temperatures compared with *N. coriiceps* when the respiratory chain was interrupted with either antimycin A or rotenone ($P < 0.05$, Fig. 1.2). Similar to rates of ROS production in untreated mitochondria, rates of ROS production were significantly higher in mitochondria of both species treated with antimycin A or rotenone at 10°C compared with 2°C ($P < 0.05$, Fig. 1.2).

1.4.3 Susceptibility to LPO

When rates of LPO were measured at a temperature that approaches physiological temperature for these animals (1°C), significant LPO was observed in mitochondrial membranes prepared from *C. aceratus* whereas LPO was undetectable in membranes from *N. coriiceps* (Fig. 1.3 A, B). Rates of LPO were significantly higher in mitochondria from *C. aceratus* at 10°C compared with 1°C ($P < 0.05$, Fig. 1.3 A, B) and higher in *N. coriiceps* at 10°C compared with 1°C, but our inability to detect LPO at 1°C in *N. coriiceps* precluded statistical analysis. Rates of LPO, measured at 10°C, were similar between *C. aceratus* and *N. coriiceps* when normalized to either protein ($P > 0.05$, Fig. 1.3 A) or phospholipid content ($P > 0.05$, Fig. 1.3 B).

1.4.4 Lipid composition

We sought to determine if differences in the composition of mitochondrial membranes might influence susceptibility to LPO. Mitochondrial membranes from *C. aceratus* had a 1.6-times higher content of phospholipid compared with *N. coriiceps* as indicated by increased levels of hydrolysable phosphate ($P < 0.05$, Appendix 1.2). Twenty-two phospholipid species were in abundances greater than 1 mol% for at least one of the study species, and the contents of nearly half (45%) of these phospholipids varied between species ($P < 0.05$, Appendix 1.2). Four phospholipids were particularly abundant (>5 mol%), and all were highly unsaturated containing at least 5 double bonds (Appendix 1.2). One was identified as major phosphatidylcholine (MPC) D16:0-20:5 or D16:1-20:4, one as lysophosphosphatidylcholine (LPC) 22:6, and two as PEs D18:1-22:6 or D18:2-22:5 and D18:0-22:6 or D18:1-22:5. All but LPC 22:6 were significantly elevated in mitochondrial membranes from *C. aceratus*. Of the four phospholipid classes quantified (cardiolipin (CL), MPC, LPC and PE), two classes were significantly different between the mitochondrial membranes of *C. aceratus* and *N. coriiceps*. Total content of MPC was 1.2-times higher in *C. aceratus* compared with *N. coriiceps* ($P < 0.05$, Table 1.2). In contrast, LPC were 1.6-times more abundant in *N. coriiceps*, relative to *C.*

aceratus ($P < 0.05$, Table 1.2). Additionally, while the UI of all LPC was 1.8- times higher in membranes from *N. coriiceps*, relative to *C. aceratus* ($P < 0.05$, Table 1.3), total membrane UI was similar between the species ($P > 0.05$, Table 1.3).

1.4.5 Antioxidant level

Maximal activities of SOD and TAOP per milligram of mitochondrial protein were equivalent between mitochondria isolated from heart ventricles of the icefish *C. aceratus* compared with the red-blooded species *N. coriiceps* ($P > 0.05$, Fig. 1.4 A, B). In contrast, when TAOP was normalized to phospholipid content, levels were 1.4-times higher in mitochondria from *N. coriiceps* compared with *C. aceratus* ($P < 0.05$, Fig. 1.4 B).

1.5 DISCUSSION

This is the first study to examine how differences in mitochondrial architecture between red- and white-blooded notothenioids impact mitochondrial function and susceptibility to oxidative stress. Mitochondria from icefishes are more tightly coupled compared with those from red-blooded species. Although potentially beneficial under normal, physiological conditions, a high degree of coupling can promote the formation of ROS when the electron transport chain is disrupted. Additionally, mitochondria from icefishes are rich in phospholipids, and our results suggest that the capacity to protect against LPO may be lower in icefishes compared with red-blooded species.

1.5.1 Mitochondria from icefishes are tightly coupled

Previous studies have suggested that high mitochondrial densities in oxidative muscle of polar fishes, rich in PUFAs (Johnston et al., 1998), may be energetically costly (Guderley, 2004) because proton leak positively scales with UI. Our data suggest

otherwise. The UI of mitochondrial membrane phospholipids is 1.9-times to 2.0-times higher in Antarctic notothenioid fishes compared with mitochondria isolated from oxidative muscle of rainbow trout (*Oncorhynchus mykiss*) acclimated to 5°C (Kraffe et al., 2007), yet mitochondria from all four Antarctic teleosts measured here were more tightly coupled compared with those from temperate teleosts. State II respiration rates were ~61-times higher in mitochondria isolated from liver of common carp (*Cyprinus carpio*) warm-acclimated to 20°C and 40-times higher in carp cold-acclimated to 8°C compared with *N. coriiceps* at 2°C when compared at a common membrane potential of 162 mV or 169 mV, respectively (Jastroch et al., 2007). Similarly, state II respiration rates were 50-times higher in mitochondria isolated from liver of cold-acclimated rainbow trout (*O. mykiss*) maintained at 4–8°C compared with *N. coriiceps* at 2°C when compared at the common membrane potential of 166 mV (Brookes et al., 1998). These differences may be overestimated because proton leak was measured at 25°C in mitochondria isolated from carp and at 20°C in rainbow trout, which in both cases was higher than the acclimation temperature of the fish (Brookes et al., 1998; Jastroch et al., 2007). However, in studies where proton leak was measured at the acclimation temperature of the animal, proton leak was still higher in the temperate species compared with Antarctic fishes. Rates of state II respiration were 10-times higher in mitochondria isolated from hearts of rainbow trout, and 11-times higher in mitochondria from oxidative muscle of rainbow trout at 15°C compared with *N. coriiceps* at 2°C when compared at a common membrane potential of 185 mV (Leary et al., 2003).

The greater proton leak in mitochondria from red-blooded notothenioids compared with icefishes may be due to their higher density of inner mitochondrial membranes per mitochondrion (Archer and Johnston, 1991; O'Brien and Sidell, 2000). Porter and colleagues determined that 69% of the difference in proton leak among mammals can be accounted for by differences in the density of inner mitochondrial membranes (Porter et al., 1996). The surface density of inner mitochondrial membranes per volume of mitochondria is 1.4-times to 1.5-times higher in heart ventricles of red-

blooded notothenioids compared with icefishes (Archer and Johnston, 1991; O'Brien and Sidell, 2000), providing a greater surface area across which protons can leak. Studies have shown that plotting state II respiration rates per milligram of cytochrome *c* oxidase (CCO) creates different leak curves compared with those obtained by plotting state II respiration rates per milligram of mitochondrial protein because the ratio between the respiratory components and other mitochondrial proteins may vary among species or tissue types (Leary et al., 2003). Although we did not measure the maximal activity of CCO in isolated mitochondria, state III respiration rates and the maximal activity of citrate synthase (CS) per milligram of mitochondrial protein are equivalent between red- and white-blooded notothenioids (data not shown), suggesting that CCO activity is likely similar as well. Thus, the differences we observed in proton leak between red- and white-blooded notothenioids would likely also be apparent when plotted as state II respiration rates per milligram of CCO.

The contribution of lipid composition to proton leak in Antarctic fishes is unclear. Omega-6 fatty acids 20:3(*n*-6) and 20:4(*n*-6), and omega-3 fatty acid 22:6(*n*-3) promote leak (Brookes et al., 1998) whereas myristic acid (14:0), palmitoleate acid (16:1(*n*-7)), oleic acid (18:1(*n*-9)), the omega-6 fatty acid 18:3(*n*-6) and the omega-3-fatty acid 18:3(*n*-3) inhibit leak (Brookes et al., 1998). Levels of the proton leak-stimulating 22:6 LPC are 2.2-times higher in *N. coriiceps* compared with *C. aceratus*, which might contribute to the greater proton leak in *N. coriiceps*. However, there are no clear differences in other proton leak-stimulating or proton leak-inhibiting phospholipids, or UI between the two species. One limitation of our study is that lipid composition was quantified in a mixture of inner and outer mitochondrial membranes. Differences in the composition of inner mitochondrial membranes among icefishes and red-blooded notothenioids might provide more insight to the extent which membrane composition mediates proton leak. Proton leak increased in all species in response to an increase in temperature. Temperature coefficient (Q_{10}) values for protein- or enzyme-mediated reactions are typically between two and three (Hochachka and Somero, 2002). The high

Q_{10} for proton leak (> 20) suggests that the increase in leak in response to temperature is not mediated by changes in activity of either UCPs or ANT (reviewed by Echtay, 2007), but rather by lipids. Moreover, proton leak was measured at 2°C and 10°C in mitochondria isolated from animals held at the same temperature (0 ± 1°C), excluding the possibility that the temperature-induced increase in proton leak could be mediated by changes in the expression of UCPs or ANT.

1.5.2 Mitochondria from icefishes have a greater potential to produce high levels of ROS than those from red-blooded notothenioids

The majority of ROS are produced by the mitochondrial respiratory chain where ~0.15% of oxygen consumed is converted to ROS (St-Pierre et al., 2002). Rates of ROS production were similar between mitochondria isolated from heart ventricles of *C. aceratus* and *N. coriiceps* at both 2°C and 10°C. Similar rates of ROS production are consistent with their similar rates of state III respiration at both 2°C and 10°C (data not shown). Rates of ROS production increased 1.5-times to 1.6-times in response to an increase in temperature from 2°C to 10°C, which is similar to values obtained in mitochondria isolated from other ectotherms. For example, ROS production increased 2.5-times under state III conditions and 2.1-times under state IV+ conditions (state IV in the presence of oligomycin) in mitochondria isolated from the gill of the Antarctic bivalve *Laternula elliptica* in response to an 8°C increase in temperature, from 1°C to 9°C (Heise et al., 2003). Similarly, ROS production increased 1.8-times under state III conditions and 1.9-times under state IV+ conditions in mitochondria isolated from mantle tissue of mud clams (*Mya arenaria*) in response to a 10°C increase in temperature, from 5°C to 15°C (Abele et al., 2002).

Disruption of electron transfer at complex I with rotenone or complex III with antimycin A resulted in a significant increase in rates of ROS production in both species. Similar to mammalian cardiac mitochondria (Turrens and Boveris, 1980), more ROS is

produced by complex III than complex I in Antarctic fishes. Rates of ROS production increased up to 6.0-times upon the addition of antimycin A, but only up to 2.9-times following the addition of rotenone. There was a much greater increase in the production of ROS in mitochondria from *C. aceratus* compared with *N. coriiceps* when electron transfer was disrupted with either rotenone or antimycin A. This difference is most likely attributable to the higher mitochondrial membrane potential of *C. aceratus* compared with *N. coriiceps* because the formation of ROS increases exponentially as membrane potential exceeds 140 mV (Kadenbach et al., 2010).

As temperature increases, rates of ROS production increase because cellular respiration increases, but ROS formation will also increase *in vivo* if tissues become hypoxic (Guzy and Schumacker, 2006). Studies in both yeast and mammals have shown that similar to the effect of antimycin A, hypoxia increases the life span of ubisemiquinone ($QH^{\cdot}$), which is formed during electron transfer between ubiquinol and complex III, and which leaks electrons to oxygen (Guzy and Schumacker, 2006; Guzy et al., 2007). There is evidence that the heart ventricle of fishes is likely one of the first tissues to become hypoxic as temperature increases (Farrell, 2002). Like many teleosts, Antarctic fishes have a serial circulation, in which the heart ventricle is oxygenated by venous blood entering the ventricular lumen (Davie and Farrell, 1991; Zummo et al., 1995). Blood oxygen content will likely decline as temperature and metabolic rate increases, resulting in inadequate oxygen delivery to the heart. Icefishes may be especially vulnerable to cardiac hypoxia because of the low oxygen-carrying capacity of their blood. Moreover, if hearts become hypoxic, the production of ROS will increase to a greater extent in icefishes because of their higher mitochondrial membrane potential and higher density of mitochondrial membranes rich in PUFAs, which promote ROS formation via the LPO cycle (reviewed in Girotti, 1998). However, the degree to which the formation of ROS is propagated by lipid radicals depends on the susceptibility of the membrane to LPO and levels of antioxidants.

1.5.3 Susceptibility of mitochondria to LPO and antioxidant capacity differs between icefishes and red-blooded notothenioids

When measured at a physiologically relevant temperature (1°C), mitochondrial membranes from heart ventricles of *C. aceratus* appeared to be more susceptible to LPO than those from *N. coriiceps*. Susceptibility to LPO is related to phospholipid content and composition; unsaturated fatty acids are more likely to become oxidized than saturated ones (Cosgrove et al., 1987), and phospholipids with ethanolamine head groups are more likely to undergo oxidation than other phospholipid classes (Wang et al., 1994). Mitochondrial membrane UI is similar between *C. aceratus* and *N. coriiceps*, and PE/PC values are somewhat lower in *C. aceratus* (1.39) than in *N. coriiceps* (1.48). Based on these lipid characteristics alone, we would predict rates of LPO to be somewhat higher in the mitochondrial membranes of *N. coriiceps* compared with those from *C. aceratus*. However, regardless of the normalization criterion employed, significant LPO was detected in mitochondrial membranes from *C. aceratus* but not in membranes from *N. coriiceps* at 1°C. These data are in agreement with previous studies (Grim et al., 2010) that showed phospholipid composition alone is not an accurate predictor of LPO susceptibility. Other properties of membranes, such as antioxidant defenses, must also be considered.

Among low molecular weight antioxidants, liposoluble vitamin E is thought to be the first line of defense against LPO (Halliwell and Gutteridge, 2007). Dunlap et al. reported that the red-blooded species *G. gibberifrons* had on average 7.4-times higher total vitamin E levels (including α-tocopherol and marine-derived tocopherol (MDT)) than *C. aceratus*, while percent MDT was generally elevated in tissues of icefishes (Dunlap et al., 2002). While MDT has a greater efficiency than α-tocopherol for inhibiting LPO at low temperature (Yamamoto et al., 2001), the relatively low abundance of this vitamin E constituent in *C. aceratus* (Dunlap et al., 2002) makes it likely that the vitamin E content of *G. gibberifrons* and perhaps in *N. coriiceps* as well, imparts a greater level of defense against LPO, despite the lower MDT content.

The activity of SOD was similar per milligram of mitochondrial protein in mitochondria isolated from heart ventricles of *C. aceratus* and *N. coriiceps*. Similarly, total antioxidant potential per milligram of mitochondrial protein was equivalent in *C. aceratus* compared with *N. coriiceps*. Nevertheless, TAOP, which includes lipid-soluble antioxidants such as vitamin E, normalized per unit of phospholipid was significantly lower in mitochondria from *C. aceratus* compared with *N. coriiceps*. We did not quantify phospholipid content in mitochondria used for measuring SOD activity, but using mean values obtained for mitochondria prepared for measuring TAOP reveals that SOD activity per unit of phospholipid was 1.5-times lower in mitochondria from *C. aceratus* compared with *N. coriiceps*. Lower levels of TAOP and SOD per unit of phospholipid may explain the greater susceptibility of mitochondrial membranes from *C. aceratus* to peroxidation compared with *N. coriiceps*. *In vivo*, the activity of CCO and CS per gram of ventricular mass is equivalent between red-blooded notothenioids and icefishes (O'Brien and Sidell, 2000), suggesting rates of production of ROS are similar per gram of tissue. However, mitochondrial densities and thus phospholipid densities are 2-times higher per gram of ventricular tissue of *C. aceratus* compared with *N. coriiceps* (Urschel and O'Brien, 2008), and the activity of both SOD and catalase per gram of tissue is 4–6-times lower in icefishes compared with red-blooded species (Cassini et al., 1993). Taken together, these data suggest mitochondrial membranes, as well as the mitochondrial-rich heart ventricles of icefishes may be at a greater risk for oxidative damage as temperature and ROS production increases. Current studies are aimed at determining whether oxidative stress is greater in hearts of icefishes compared with red-blooded species exposed to elevated temperatures.

1.5.4 Conclusions

Current climate trends suggest a warmer future for fishes inhabiting the Southern Ocean and particularly the WAP region (Clarke et al., 2007). Elevations in temperature will be challenging for Antarctic notothenioids, which seem to have a reduced capacity to adjust

to fluctuations in temperature compared with temperate and more Northern fish species (Jin and DeVries, 2006; Buckley and Somero, 2009). Oxidative stress is one challenge imposed by elevated temperatures (Parihar and Dubey, 1995; Heise et al., 2006; Lushchak and Bagnyukova, 2006a; Lushchak and Bagnyukova, 2006b). Icefishes may be more susceptible to oxidative damage because of their greater capacity to produce ROS under some conditions, which may be propagated by high densities of polyunsaturated phospholipids within cardiac muscle. We do not know if small, incremental increases in temperature, as would be experienced during global warming might induce antioxidant defenses, or if similar to the heat-shock response (Hofmann et al., 2000), Antarctic fishes have lost the ability to elevate antioxidant defenses and combat oxidative stress. Future studies will address this question.

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1.8 FIGURES

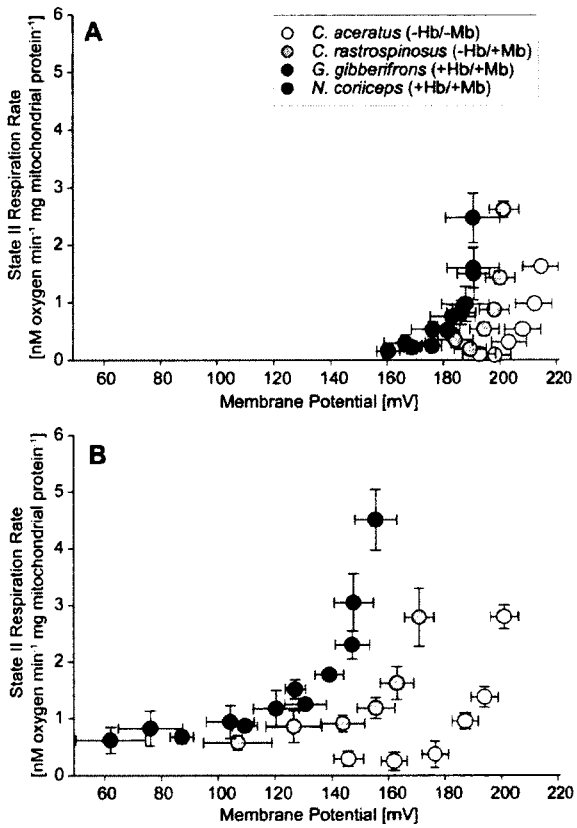

Figure 1.1 Proton leak in mitochondria of Antarctic notothenioid fishes

Proton leak in mitochondria isolated from heart ventricle of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps* at 2°C (A) and 10°C (B). State II respiration rates were quantified with succinate as substrate and plotted against membrane potential obtained by titration with the complex II inhibitor, malonate. N = 4-5.

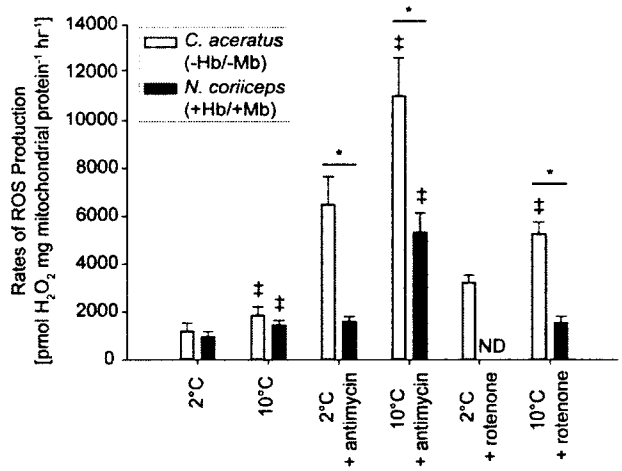

Figure 1.2 Rates of ROS production in mitochondria of Antarctic notothenioid fishes

Rates of ROS production in mitochondria isolated from heart ventricle of *C. aceratus* and *N. coriiceps* at 2°C and 10°C. Rates of ROS production were quantified by monitoring the formation of resorufin in the presence and absence of the respiratory chain inhibitors antimycin A and rotenone. ND = not detected. N = 10. Significant differences between species at common temperatures and treatments are indicated by asterisks. Significant differences within a species at different temperatures and treatments are indicated by double daggers. $P < 0.05$.

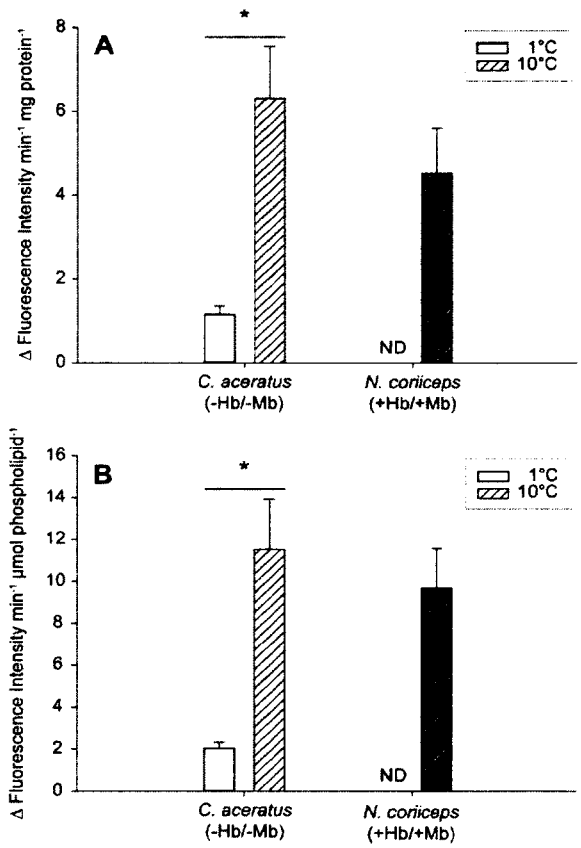

Figure 1.3 Rates of LPO in mitochondria of Antarctic notothenioid fishes

Rates of LPO in mitochondrial membranes of *C. aceratus* and *N. coriiceps* at 1°C and 10°C, normalized to protein content (A) and phospholipid content of mitochondrial membranes (B). LPO was induced in membrane preparations using hydroxyl radicals produced by the Fenton reaction between Cu^{2+} and cumene hydroperoxide. ND = not detected. N = 6. Significant differences within a species at different temperatures are indicated by asterisks. $P < 0.05$.

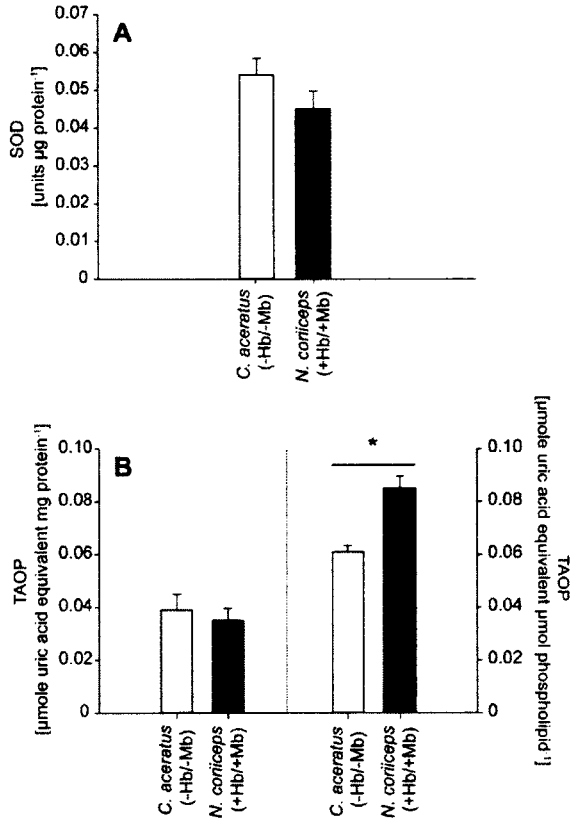

Figure 1.4 Antioxidant capacities of mitochondria of Antarctic notothenioid fishes
Antioxidant capacity of isolated mitochondria from red- and white-blooded notothenioid fishes. (A) Activity of SOD was measured at 5°C in mitochondria isolated from heart ventricles of *C. aceratus* and *N. coriiceps*. N = 6. (B) Total antioxidant power (TAOP) of mitochondria from *C. aceratus* and *N. coriiceps* normalized per mg mitochondrial protein or phospholipid content of mitochondrial membranes. N = 6. Significant differences between species are indicated by asterisks. $P < 0.05$.

1.9 TABLES

Table 1.1 Rates of mitochondrial oxygen consumption at a common membrane potential in Antarctic notothenioid fishes

State II oxygen consumption [nmol oxygen min^{-1} mg $protein^{-1}$] of isolated mitochondria of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps* at a common membrane potential at 2°C or 10°C.

	Oxygen consumption [nmol oxygen min^{-1} mg $protein^{-1}$]	
	at 2°C and 190 mV	**at 10°C and 147 mV**
C. aceratus	0.093*	0.249*
C. rastrospinosus	0.409*	0.910*
G. gibberifrons	1.598 ± 0.348	3.042 ± 0.513
N. coriiceps	1.499 ± 0.456	2.293 ± 0.251

* oxygen consumption determined by interpolation

Values are expressed as mean ± s.e.m. unless determined by interpolation. N = 4-5.

Table 1.2 Relative abundance of phospholipid classes in mitochondrial membranes of Antarctic notothenioid fishes

Relative abundance of selected phospholipid classes in mitochondrial membranes of *C. aceratus* and *N. coriiceps*

	C. aceratus	*N. coriiceps*
CL	5.31 ± 0.30	6.37 ± 0.55
MPC	34.77 ± 1.00	30.14 ± 1.42*
LPC	11.59 ± 1.76	18.82 ± 1.93*
PE	48.33 ± 1.74	44.67 ± 1.59
Total	**100%**	**100%**

CL – cardiolipin, MPC – major phosphatidylcholine, LPC – lysophosphatidylcholine, PE – phosphatidylethanolamine. Values are expressed as mean ± s.e.m. N = 6. Significant differences in abundance of individual phospholipid classes between species are indicated with asterisks. $P < 0.05$.

Table 1.3 Unsaturation indices of phospholipid classes in mitochondrial membranes of Antarctic notothenioid fishes

Unsaturation indices (UI) of select phospholipid classes in mitochondrial membranes of *C. aceratus* and *N. coriiceps*

	C. aceratus	*N. coriiceps*
CL	71.70 ± 4.01	78.29 ± 7.17
MPC	188.95 ± 6.44	172.05 ± 8.52
LPC	55.82 ± 8.39	97.87 ± 10.50*
PE	299.19 ± 10.00	271.69 ± 9.57
Total	**615.65 ± 3.84**	**619.90 ± 2.88**

UI calculated following the modifications of Hulbert et al. (Hulbert et al, 2007) by Grim et al. (Grim et al, 2010). CL – cardiolipin, MPC – major phosphatidylcholine, LPC – lysophosphatidylcholine, PE – phosphatidylethanolamine. Values are expressed as mean ± s.e.m. N = 6. Significant differences in abundance of individual phospholipids between species are indicated with asterisks. $P < 0.05$.

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1.11 APPENDICES

Appendix 1.1 Mitochondrial matrix volume and non-specific $TPMP^+$ binding in Antarctic notothenioid fishes

Mitochondrial matrix volume [μl mg protein^{-1}] and correction factors for non-specific $TPMP^+$ binding [mg protein^{-1}] used for quantification of proton leak in isolated mitochondria of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps*.

	C. aceratus	***C. rastrospinosus***	***G. gibberifrons***	***N. coriiceps***
Matrix volume	1.52	0.91*	0.51**	0.51
Unspecific $TPMP^+$ binding, 2°C	0.896 ± 0.046	0.920 ± 0.044	0.991 **	0.991 ± 0.005
Unspecific $TPMP^+$ binding, 10°C	0.974 ± 0.021	0.986 ± 0.001	0.964 **	0.964 ± 0.014

* determined based on stereological measurements

** assumed to be similar to *N. coriiceps*

Non-specific $TPMP^+$ binding is expressed as mean ± s.e.m. N = 2-6

Appendix 1.2 Abundance of individual phospholipids in mitochondrial membranes of Antarctic notothenioid fishes

Abundance of individual phospholipid species in mitochondrial membranes of *C. aceratus* and *N. coriiceps*

	Possible Species	***C. aceratus***	***N. coriiceps***
CL	18:2-18:0-22:6-22:6		
	22:6-22:6-18:1-18:1	1.12 ± 0.08	0.67 ± 0.10*
	22:6-20:4-20:3-18:1		
CL	20:4-20:4-22:6-22:5		
	18:2-22:6-22:6-22:5	0.85 ± 0.07	0.99 ± 0.07
	22:6-22:6-22:6-18:1		
	22:6-22:6-20:4-20:3		
MPC	D16:0-18:1	3.66 ± 0.09	1.79 ± 0.10*
	D16:1-18:0		
MPC	D16:1-20:5	2.37 ± 0.08	1.75 ± 0.26
MPC	D16:0-20:5	6.17 ± 0.42	3.83 ± 0.35*
	D16:1-20:4		
MPC	D16:1-22:6	2.24 ± 0.22	1.85 ± 0.22
	D18:2-20:5		
MPC	D16:0-22:6		
	D18:1-20:5	16.76 ± 1.12	17.81 ± 0.90
	D18:2-20:4		
MPC	D18:1-22:6	2.53 ± 0.26	2.18 ± 0.20
MPC	D18:0-22:6	1.04 ± 0.06	0.93 ± 0.05
	D18:1-22:5		

Appendix 1.2 Abundance of individual phospholipids in mitochondrial membranes of Antarctic notothenioid fishes continued

	Possible Species	*C. aceratus*	*N. coriiceps*
LPC	20:5	2.63 ± 0.44	2.11 ± 0.27
LPC	22:6	6.11 ± 0.91	13.33 ± 1.56*
PE	D16:0-20:5	1.48 ± 0.15	1.79 ± 0.20
	D16:1-20:4		
PE	P16:0-22:6	1.35 ± 0.05	1.36 ± 0.08
	P18:1-20:5		
	A18:2-20:5		
	P18:2-20:4		
PE	P18:1-20:4	1.36 ± 0.09	1.84 ± 0.10*
	P18:0-20:5		
	A18:1-20:5		
	A16:0-22:6		
	P16:0-22:5		
	P18:2-20:3		
	A16:1-22:5		
	A18:2-20:4		
PE	D16:1-22:6	1.20 ± 0.06	1.57 ± 0.06*
	D18:2-20:5		
PE	D16:0-22:6	13.90 ± 1.00	13.72 ± 1.05
	D18:1-20:5		
	D18:2-20:4		
PE	D18:1-20:4	1.46 ± 0.07	1.88 ± 0.13*
	D18:0-20:5		
	D16:0-22:5		

Appendix 1.2 Abundance of individual phospholipids in mitochondrial membranes of Antarctic notothenioid fishes continued

	Possible Species	*C. aceratus*	*N. coriiceps*
PE	D18:0-20:4		
	D18:1-20:3	0.80 ± 0.06	1.01 ± 0.07
	D16:0-22:4		
PE	P18:1-22:6		
	A18:2-22:6	2.07 ± 0.08	1.41 ± 0.04*
	P18:2-22:5		
PE	P18:0-22:6		
	P18:1-22:5	1.32 ± 0.06	1.41 ± 0.11
	A18:1-22:6		
	A18:2-22:5		
PE	D18:1-22:6	12.57 ± 0.50	8.95 ± 0.67
	D18:2-22:5		
PE	D18:0-22:6	6.86 ± 0.40	5.39 ± 0.48*
	D18:1-22:5		
Total phosphate (mM)		**18.07 ± 1.61**	**11.38 ± 0.89***

Only phospholipids present in amounts ≥ 1 mol% for at least one of the species are shown. CL – cardiolipin, MPC – major phosphatidylcholine, LPC – lysophosphatidylcholine, PE – phosphatidylethanolamine. D – phosphatidyl (ester linked), A – plasmanyl (alkyl ether linked), and P – plasmenyl (vinyl ether linked). Values are expressed as mean ± s.e.m. N = 6. Significant differences in abundance of individual phospholipids between species are indicated with asterisks. $P < 0.05$

CHAPTER 2: ELEVATION IN TEMPERATURE CAUSES OXIDATIVE STRESS IN HEARTS OF WHITE- BUT NOT RED-BLOODED ANTARCTIC NOTOTHENIOID FISHES[1]

[1] Mueller, I. A., Devor, D. P, Grim, J. M., Beers, J. M., Crockett, E. L. and O'Brien, K. M. 201X. Elevation in temperature causes oxidative stress in hearts of white- but not red-blooded Antarctic notothenioid fishes. Submitted to *Journal of Experimental Biology*

2.1 SUMMARY

Antarctic icefishes have a significantly lower critical thermal maximum (CTmax) compared to most red-blooded notothenioids. We hypothesized that icefishes may be more vulnerable to oxidative stress as temperature increases compared to red-blooded notothenioids, contributing to their lower thermal tolerance. Oxidative muscles of icefishes have high volume densities of mitochondria, rich in polyunsaturated fatty acids, which can promote the production of reactive oxygen species (ROS). Moreover, icefishes have lower levels of antioxidants compared to red-blooded species. To test our hypothesis, we measured levels of oxidized proteins and lipids as well as transcript level and maximal activity of the antioxidants superoxide dismutase and catalase in heart ventricles and oxidative pectoral adductor muscle of icefishes and red-blooded notothenioids held at 0°C and exposed to their CTmax. Levels of oxidized proteins and lipids increased in heart ventricle of icefishes but not in red-blooded species in response to warming, and not in pectoral adductor muscle of either species. Thus, oxidative damage in heart ventricles may contribute to the reduced thermal tolerance of icefishes. Furthermore, neither transcript level nor activity of antioxidants was elevated in any tissue or species in response to exposure to CTmax, suggesting notothenioids may have lost the ability to regulate antioxidant levels.

2.2 INTRODUCTION

The Southern Ocean is one of the most thermally stable and cold environments on Earth (Eastman, 1993b). Water temperatures in the Ross Sea hover near -1.9°C year round, and even in the more northerly region of the Western Antarctic Peninsula (WAP), water temperatures fluctuate minimally between -1.8°C and +2°C (Eastman, 1993a; Hofmann and Klinck, 1998; Jacobs et al., 2002). The future Southern Ocean, however, may look

quite different, as the WAP is one of the fastest warming regions on Earth (Vaughan et al., 2003; Meredith and King, 2005; Turner et al., 2005). Annual near-surface air temperatures have risen by 0.56°C decade^{-1} since 1950, resulting in warming of the upper water column by more than 1°C (Vaughan et al., 2003; Meredith and King, 2005; Turner et al., 2005). Rapid warming in the WAP has altered species composition, abundance of phytoplankton and zooplankton communities, and impacted higher trophic levels, especially penguin and sea bird populations (Moline et al., 2004; Ducklow et al., 2007; Montes-Hugo et al., 2009; reviewed in Schofield et al., 2010). Less is known about the impact of climate change on notothenioid fishes, the dominant suborder of fishes inhabiting the Southern Ocean (Eastman, 1993c). However, empirical evidence to date suggests that notothenioids have a limited capacity to endure elevations in temperature.

The upper incipient lethal temperature (UILT), the temperature at which 50% of fish survive for one week, is only 6°C for notothenioids captured in McMurdo Sound (Somero and DeVries, 1967). The critical thermal maximum (CTmax), a second metric for assessing thermal tolerance, measured as the temperature at which fish lose the ability to right themselves as water temperature increases, is between 12.0°C and 14.5°C for notothenioids captured in McMurdo Sound, and slightly higher for fishes in the WAP, ranging between 13.3°C and 17.1°C (Beers and Sidell, 2011; Bilyk and Devries, 2011). Both the UILT and CTmax are markedly lower in Antarctic notothenioids compared to temperate fishes. For example, the UILT of salmonids is 24-27°C (Elliott, 1991), and the CTmax of rainbow trout (*Oncorhynchus mykiss*) and common eelpout (*Zoarces viviparous*), ranges between 27°C and 34°C (Zakhartsev et al., 2003; Patra et al., 2007).

The physiological underpinnings of thermal tolerance are largely unknown but likely attributable to multiple factors, including neuronal function, metabolic scope, and sensitivity to oxidative stress. Oxygen consumption in brain slices, but not in gills of notothenioids is impaired at temperatures close to their UILT, suggesting a link between neuronal function and thermal tolerance (Somero and DeVries, 1967). Additionally, as

temperature rises, the oxygen-carrying capacity of blood decreases and oxygen demand increases, resulting in a mismatch between oxygen supply and demand, which may result in hypoxia. Consistent with this, studies have shown that as temperature increases, the onset of cardiac arrhythmia, and increases in blood acidosis and blood lactate levels are correlated with a decrease in venous blood oxygen content in Atlantic cod (*Gadus morhua*) and Chinook salmon (*Oncorhynchus tshawytscha*) (Lannig et al., 2004; Clark et al., 2008). Venous P_{O2} is approximately 1.5-times lower and venous oxygen content (ml dl^{-1}) approximately 2.5-times lower in Chinook salmon exposed to 24°C compared to animals at 13°C (Clark et al., 2008). Similarly, venous P_{O2} decreases approximately 2.5-times in Atlantic cod as temperature increases from 5°C to 15°C (Lannig et al., 2004). Oxidative stress also increases with temperature and may contribute to defining an organism's window of thermal tolerance. As temperature increases, rates of production of reactive oxygen species (ROS) increase *in vitro* in mitochondria isolated from notothenioids and other ectotherms, including mud clams (*Mya arenaria*), Antarctic bivalves (*Laternula elliptica*) and lugworms (*Arenicola marina*) (Abele et al., 2002; Heise et al., 2003; Keller et al., 2004; Mueller et al., 2011). An increase in ROS production damages major biological macromolecules, including nucleic acids, proteins and lipids unless organisms simultaneously elevate protective antioxidant levels (reviewed in Halliwell, 2011).

Antarctic icefishes (family Channichthyidae, suborder Notothenioidei), lacking the oxygen-binding protein hemoglobin (Hb), may be especially vulnerable to elevations in temperature (Ruud, 1954; Beers and Sidell, 2011). The CTmax of the icefishes *Chionodraco rastrospinosus* and *Chaenocephalus aceratus* is 13.3°C and 13.9°C, respectively, which is significantly lower than the CTmax of the red-blooded species *Gobionotothen gibberifrons* (15.5°C) and *Notothenia coriiceps* (17.1°C) (Beers and Sidell, 2011). Several factors may contribute to the low thermal tolerance of icefishes. The oxygen-carrying capacity of the blood of icefishes is only 10% that of red-blooded notothenioids (Ruud, 1954). As temperature increases, the oxygen-carrying capacity of

icefish blood will decline more precipitously compared to red-blooded species because the solubility of oxygen in water is negatively correlated with temperature. Consequently, icefishes are more likely to experience a mismatch between oxygen supply and demand at lower temperatures than red-blooded notothenioids. Additionally, icefishes may be more vulnerable to oxidative stress as temperature increases than red-blooded species. Although rates of production of ROS are similar in mitochondria isolated from red- and white-blooded notothenioids, the density of unsaturated mitochondrial phospholipids, which can promote the formation of ROS, is higher in aerobic muscles of icefishes compared to red-blooded fishes (O'Brien and Sidell, 2000; O'Brien et al., 2003; Mueller et al., 2011). Moreover, when the respiratory chain is disrupted, which occurs during hypoxic events, mitochondria from icefishes produce more ROS than red-blooded fishes (Mueller et al., 2011). Compounding the effect, icefishes have lower levels of antioxidants compared to red-blooded species. Maximal activities of the antioxidants superoxide dismutase (SOD) and catalase are between 1.4-times and 6.0-times lower in liver, heart and muscle of the icefish *Chionodraco hamatus* compared to the red-blooded notothenioid *Trematomus bernacchii* (Cassini et al., 1993). Similarly, maximal activities of SOD is 6.5-times lower in liver of the icefishes *C. aceratus* and *Pseudochaenichthys georgianus* compared to the red-blooded species *G. gibberifrons* and *N. coriiceps* (Witas et al., 1984). Furthermore, levels of vitamin E and its derivatives, which protect membranes from peroxidation, are up to 13.0-times lower in liver and 1.7-times lower in the pectoral adductor muscle of *C. aceratus* compared to *G. gibberifrons* (Dunlap et al., 2002).

We tested the hypothesis that icefishes are more vulnerable to oxidative stress compared to red-blooded notothenioids as temperature increases. Levels of oxidized proteins and phospholipids, as well as transcript levels and activity of the antioxidants SOD and catalase were quantified in heart ventricles and oxidative pectoral adductor muscle of icefishes (*C. aceratus*, *C. rastrospinosus*) and red-blooded notothenioids (*G. gibberifrons*, *N. coriiceps*) maintained at ambient temperature and exposed to their

CTmax. Our results indicate that icefishes but not red-blooded notothenioids experience oxidative stress during warming, which might contribute to their reduced thermal tolerance.

2.3 MATERIAL AND METHODS

2.3.1 Animal and tissue collection

C. aceratus (Lönnberg), *C. rastrospinosus* (DeWitt and Hureau), *G. gibberifrons* (Lönnberg) and *N. coriiceps* (Richardson) were caught during the austral fall of 2009 in Dallmann Bay (64°08'S, 62°40'W), Antarctica. Animals were captured using an otter trawl or baited traps deployed from the ARSV *Laurence M. Gould* and transferred to the US Antarctic research station, Palmer Station, where animals were maintained in circulating seawater tanks at 0 ± 0.5°C. The CTmax was measured (*C. aceratus*, 13.9 ± 0.4°C; *C. rastrospinosus*, 13.3 ± 0.2°C; *G. gibberifrons*, 15.5 ± 0.2°C; *N. coriiceps*, 17.1 ± 0.2°C) as described by Beers and Sidell (Beers and Sidell, 2011). Briefly, two or three individuals were transferred into insulated, 700 l experimental tanks containing seawater at ambient temperature. The CTmax was then determined by increasing the water temperature in the tank by 3.6°C h^{-1} until fishes lost their ability to right themselves. Control animals were maintained in tanks at 0°C. Animals were anesthetized in MS-222 (1:7500 in seawater) and killed by transection of the spinal cord. Tissues were quickly excised, frozen in liquid nitrogen and stored at -80°C until further use. All procedures were approved by the University of Alaska Fairbanks Institutional Animal Care Committee (134774-2).

2.3.2 Protein carbonylation

Levels of protein carbonyls were quantified in ventricle and pectoral adductor muscle of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps* as described by Levine et al. (Levine et al., 2000). Briefly, tissues (21-49 mg) were homogenized in 9 volumes (v/w) of ice-cold 0.5 mol l^{-1} potassium phosphate buffer pH 7.8 and incubated with 70 μl of 10% streptomycin in 50 mmol l^{-1} HEPES, pH 7.2 for 15 min at room temperature to precipitate nucleic acids. Homogenates were then centrifuged for 10 min at 9,300 ***g*** at 4°C. Supernatant was decanted and incubated with 1 ml of ice-cold acetone for 30 min at -20°C to precipitate proteins. Proteins were then pelleted by centrifugation (15 min, 16,100 ***g***, 4°C) and washed with 1 ml of ice-cold 80% acetone. The final protein pellet was resuspended in 100 μl of 6 mol l^{-1} guanidinium HCl, pH 2.5 and divided into four equal aliquots. Two aliquots were incubated with 35 μl of 6 mol l^{-1} guanidinium HCl, pH 2.5 and two aliquots were incubated with of 35 μl of 10 mmol l^{-1} dinitrophenylhydrazine (DNPH) in 6 mol l^{-1} guanidinium HCl, pH 2.5 for 30 min at room temperature immediately prior to separating proteins using HPLC. DNPH reacts with protein carbonyls, forming hydrazones, which were detected at 366 nm using Waters 1525 HPLC equipped with a Waters 2296 Photodiode Array Detector (Waters, Milford, MA, USA). Proteins were separated on Zorbax 450 and Zorbax 250 gel filtration columns (Waters, Milford, MA, USA) arranged in series and eluted with 6 mol l^{-1} guanidinium HCl, pH 2.5 at a flow-rate of 1 ml min^{-1}. All measurements were made in duplicate in 7-11 individuals per species and temperature treatment. Levels of protein carbonyls were quantified by integrating the area under the curve detected at 366 nm and normalized to the total protein content, which was quantified by integrating the area under the curve detected at 280 nm. Background was measured in aliquots untreated with DNPH and subtracted from protein carbonyls detected in DNPH-treated aliquots as follows:

$$\text{mmol carbonyls mol}^{-1}\text{ protein} = \frac{C_1 * A_{366}}{C_2 * (A_{280} - (0.43 * A_{366})} - \frac{C_1 * A_{366}}{C_2 * A_{280}} * 1000 \qquad \text{(Eq. 2.1)}$$

where A_{366} represents the integrated area of peaks detected at 366 nm, A_{280} represents the integrated area of peaks detected at 280 nm, C_1 represents the extinction coefficient of average proteins (ε = 50,000 L mol^{-1}) and C_2 represent the extinction coefficient of hydrazones (ε = 22,000 L mol^{-1}).

2.3.3 Lipid peroxidation

Thiobarbituric acid-reactive substances (TBARS), expressed as levels of malondialdyde (MDA), were measured in heart ventricle and pectoral adductor muscles of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps* according to Uchimaya and Mihara (Uchiyama and Mihara, 1978). Tissues were diced in ice-cold 1.15% KCl (10%, w/v) and then homogenized with five short bursts using Tekmar Tissuemizer (Teledyne Tekmar, Cincinnati, OH, USA). Further homogenization was done by three passes in Ten-Broeck ground glass homogenizers (Wheaton, Milliville, NJ, USA). After addition of 1% phosphoric acid and 0.6% thiobarbituric acid, sample homogenates were heated to 95°C for 45 minutes (1.4 ml total volume). Samples were subsequently cooled to room temperature before adding 1.1 ml butanol. After vortexing for 5 sec, samples were centrifuged at 1000 ***g*** for 10 min, top layers were decanted, and absorbance of samples was measured at 535 nm and 520 nm (with the difference between the two indicating the MDA content) using a Hewlett Packard 8453 spectrophotometer (Hewlett Packard, Palo Alto, CA, USA). All measurements were made in at least duplicate in 7-14 individuals per species and temperature treatment. An MDA standard curve was prepared and run alongside samples to quantify MDA content in samples.

2.3.4 Isolating RNA

Total RNA was isolated from heart ventricles of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps* using the RNeasy Fibrous Tissue Mini Kit (QIAGEN, Valencia, CA, USA) with minor modifications from the manufacturer's protocol. The

tissue homogenate was treated twice with DNase I at room temperature for 25 min and 20 min, respectively. Quality of the RNA was verified with a Nanodrop ND-1000 spectrophotometer (ThermoScientific, Fisher, Pittsburgh, PA, USA). Samples having an absorbance ratio of 260 nm-to-230 nm > 1.6, and an absorbance ratio of 260 nm-to-280 nm > 1.8 were used for further analyses. Integrity of RNA was verified by separation on a 2% agarose gel stained with ethidium bromide.

RNA was transcribed into complementary DNA (cDNA) using TaqMan Reverse Transcriptase Reagents (Applied Biosystems, Carlsbad, CA, USA). Reverse transcriptase was omitted in control reactions to ensure that genomic DNA was not amplified during quantitative real-time PCR (qRT-PCR).

2.3.5 Cloning and sequencing of SOD1, SOD2, CAT and EF-1α

Degenerate primers were used to amplify partial cDNA sequences of superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), catalase (CAT) and elongation factor 1α (EF-1α). Partial sequences of SOD1, SOD2 and CAT were amplified in all four species, while EF-1α was only amplified in *G. gibberifrons* as partial sequences for the other species were obtained previously (Gen bank, EU857824, EU857825, 857826) (Urschel and O'Brien, 2008). Degenerate primers were designed over conserved regions using CODEHOP (Rose et al., 2003) (Table 2.1). SOD1, SOD2, CAT and EF-1α sequences were amplified with an iCycler (Bio-Rad, Hercules, CA, USA) using a touchdown protocol with annealing temperatures between 65°C and 55°C. PCR products were separated on 2% agarose gels stained with ethidium bromide. cDNA fragments of appropriate size were excised, isolated using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA), and cloned into *Escherichia coli* using TOPO TA Cloning Kit with pCR2.1-TOPO vector and TOP10 chemically competent cells (Invitrogen, Carlsbad, CA, USA). Transformed colonies were selected based on ampicillin resistance and blue/white screening on LB plates (10 mg ml^{-1} bactotryptone, 5 mg ml^{-1} yeast extract, 5 mg ml^{-1}

NaCl, 1 mg ml^{-1} glucose, 15 mg ml^{-1} agar, 50 μg ml^{-1} ampicillin) supplemented with 64 μg ml^{-1} 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-GAL). Transformed *E. coli* were grown overnight in LB media (10 mg ml^{-1} bactotryptone, 5 mg ml^{-1} yeast extract, 5 mg ml^{-1} NaCl, 1 mg ml^{-1} glucose) supplemented with 50 μg ml^{-1} ampicillin at 37°C in a shaking water bath. Plasmids were isolated using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA, USA) and prepared for sequencing with the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). Products were purified with Centri-Sep columns (Princeton Separations, Adelphia, NJ, USA) packed with Sephadex G-50 gel (Sigma-Aldrich, St Louis, MO, USA) and sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). Sequence identity was determined by searching for homologous sequences in the NCBI nucleotide data base (http://blast.ncbi.nlm.nih.gov). Partial sequences for SOD1, SOD2, CAT and EF-1α are shown in Appendices 2.1-2.16.

2.3.6 Quantitative real-time PCR

Gene-specific primers were designed for qRT-PCR using Primer Express v2.0 software (Applied Biosystems, Carlsbad, CA, USA) (Table 2.1). Either the forward or the reverse primer of each primer set annealed over a splice site to ensure that genomic DNA was not amplified (Appendices 2.1-2.16). Primers for amplifying EF-1α were determined previously (Urschel and O'Brien, 2008). Transcript levels of SOD1, SOD2, CAT and EF-1α were quantified using qRT-PCR and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) as described by Orczewska et al. (Orczewska et al., 2010). Each reaction contained 1X Power SYBR Green PCR Master Mix, 300 μM forward primer, 300 μM reverse primer and 7.5 ng cDNA. All measurements were made in triplicate in 6-8 individuals per species and treatment. A standard curve, generated by serially-diluting cDNA pooled from all species, was used to determine relative transcript levels of SOD1, SOD2, CAT and EF-1α. The relative transcript level of SOD1, SOD2 and CAT were normalized to transcript levels of EF-1α,

which was identified as a suitable housekeeping gene using BestKeeper v1 software (Pfaffl et al., 2004) as described previously by Orczewska et al. (Orczewska et al., 2010).

2.3.7 Activity of SOD (EC 1.15.1.1) and catalase (EC 1.11.1.6)

Maximal activities of SOD and catalase were quantified in ventricle and pectoral adductor muscle of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps* held at either 0°C or exposed to their CTmax using a Perkin Elmer Lambda 25 spectrophotometer (Perkin-Elmer, Waltham, MA, USA) equipped with a refrigerated, circulating water bath.

Maximal activity of SOD was quantified at 5 ± 0.5°C by monitoring the reduction of cytochrome *c* at 550 nm (McCord and Fridovich, 1969; Crapo et al., 1978). Briefly, tissues were homogenized in 9 volumes (v/w) of ice-cold 50 mmol l^{-1} potassium phosphate, 0.1 mmol l^{-1} EDTA, pH 7.8. The reduction of 0.01 mmol l^{-1} acetylated cytochrome *c* was measured in the presence of 0.05 mmol l^{-1} xanthine, 0.01 mmol l^{-1} KCN and xanthine oxidase (XO). The final concentration of XO was determined each day to obtain a reduction rate of cytochrome *c* of 0.02 OD min^{-1}. One unit of SOD activity is defined as the amount of SOD needed to achieve 50% inhibition of the reduction of cytochrome *c*. Homogenates were diluted until a reduction rate of 0.01 ± 0.008 OD min^{-1} was achieved. All measurements were made in duplicate in 6 individuals per species and temperature treatment. Activity of SOD was expressed as Units g wet $tissue^{-1}$.

Maximal activity of catalase was quantified at 5 ± 0.5°C by monitoring the decomposition of hydrogen peroxide at 240 nm as described by Beers and Sizer (Beers and Sizer, 1952). Briefly, tissues were homogenized in 9 volumes (v/w) of 50 mmol l^{-1} phosphate buffer, pH 7.8. Background rates were determined for 2 min by monitoring rates of hydrogen peroxide decomposition in a reaction mixture containing 10 μl or 25 μl

tissue homogenate in 50 mmol l^{-1} phosphate buffer, pH 7.8 in a final volume of 1 ml. Enzyme reactions were initiated by adding a final concentration of 11 mmol l^{-1} hydrogen peroxide to the reaction mixture. All measurements were made in triplicate in 6-8 individuals per species and temperature treatment. Activity of catalase was expressed as μmol min^{-1} g wet $tissue^{-1}$.

2.3.8 Statistical analysis

Significant differences in levels of protein and lipid oxidation, transcript levels of SOD1, SOD2 and CAT, and enzyme activity of SOD and catalase between species and at a common temperature were determined using an ANOVA followed by a post-hoc Tukey-Kramer honestly significant difference (HSD) test. Data were log transformed as necessary to maintain assumptions of normality. Data not meeting the assumption of normality after log transformation were compared using a Wilcoxon test followed by a post-hoc Tukey-Kramer HSD test. Significant differences in levels of protein and lipid oxidation, transcript levels of SOD1, SOD2 and CAT, and enzyme activity of SOD and catalase between temperature treatments within a species, and between tissues within species and temperature treatments were determined using a Student's *t*-test. All data were analyzed using JMP7 (SAS, Cary, NC, USA) with significance set at $P < 0.05$ unless otherwise noted. All data are expressed as mean ± s.e.m.

2.4 RESULTS

2.4.1 Levels of protein oxidation

Levels of protein carbonyls increased 28-times in ventricles of *C. aceratus* exposed to their CTmax ($P < 0.05$, Fig. 2.1 A) but remained constant in all other species ($P > 0.05$, Fig. 2.1 A). In animals held at ambient temperature, levels of protein carbonyls were

lowest in ventricles of *C. aceratus* (0.34 ± 0.33 mmol carbonyl mol protein^{-1}), intermediate in *C. rastrospinosus* (8.8 ± 2.1 mmol carbonyl mol protein^{-1}) and *G. gibberifrons* (9.4 ± 3.0 mmol carbonyl mol protein^{-1}) and highest in *N. coriiceps* (27.5 ± 5.0 mmol carbonyl mol protein^{-1}) ($P < 0.05$, Fig. 2.1 A).

Levels of protein carbonyls did not increase in response to exposure to CTmax in pectoral adductor muscle of any species and were similar among all four species when held at 0°C ($P > 0.05$, Fig. 2.1 B). Levels of carbonyls ranged between 9.4 ± 3.5 mmol carbonyls mol protein^{-1} (*C. aceratus*) and 21.2 ± 6.1 mmol carbonyls mol protein^{-1} (*N. coriiceps*).

Levels of carbonyls were significantly higher in pectoral adductor muscle of *C. aceratus* compared to heart ventricle ($P < 0.05$), but were equivalent between ventricles and pectoral adductor muscles in all other species ($P > 0.05$).

2.4.2 Levels of oxidized lipids

Levels of MDA increased 1.4-times in hearts of both *C. aceratus* and *C. rastrospinosus* in response to exposure to CTmax ($P < 0.05$) but did not increase in hearts of red-blooded species ($P > 0.05$, Fig. 2.2 A).

Levels of MDA were not significantly different between heart ventricles of red- and white-blooded species held at 0°C and ranged from 8.8 ± 1.0 nmol MDA g wet tissue^{-1} (*N. coriiceps*) to 12.9 ± 0.9 nmol MDA g wet tissue^{-1} (*C. rastrospinosus)* ($P > 0.05$, Fig. 2.2 A).

Levels of MDA in pectoral adductor muscles were not affected by exposing animals to their CTmax ($P > 0.05$, Fig. 2.2 B). Levels of MDA were similar in pectoral adductor muscle of *C. aceratus*, *C. rastrospinosus* and *G. gibberifrons*, ranging between 16.5 ± 0.9

nmol MDA g wet tissue^{-1} to 22.9 ± 2.5 nmol MDA g wet tissue^{-1} ($P > 0.05$), but were up to 2.8-times higher in *N. coriiceps* ($P < 0.05$, Fig. 2.2 B).

Levels of MDA were significantly higher in the pectoral adductor muscle of all four species compared to their heart ventricle ($P < 0.05$).

2.4.3 Transcript level of antioxidants

Transcript levels of neither SOD1, SOD2 nor CAT were up-regulated in hearts of any species in response to exposure to CTmax (Fig. 2.3 A-C). However, transcript levels of SOD1 decreased in hearts of both *C. aceratus* and *C. rastrospinosus* and transcript levels of SOD2 also decreased in hearts of *C. aceratus* in response exposure to CTmax ($P < 0.05$, Fig 2.3 A, B).

In general, transcript levels of antioxidants were similar between hearts of red-blooded species, and similar between hearts of icefishes, yet higher in red-blooded species compared to white-blooded ones. Transcript levels of SOD1 were 1.7-times to 2.3-times higher in ventricle of *G. gibberifrons* and *N. coriiceps* compared to *C. aceratus* and *C. rastrospinosus* ($P < 0.05$, Fig 2.3 A). Similarly, transcript levels of SOD2 were 2.8-times to 4.5-times higher in ventricle *G. gibberifrons* and *N. coriiceps* compared *C. aceratus* and *C. rastrospinosus* ($P < 0.05$, Fig. 2.3 B). Transcript levels of CAT were 4.8-times to 5.6-times higher in ventricles of the two red-blooded species compared to *C. aceratus*, and 1.8-times higher in the ventricle of *C. rastrospinosus* compared to red-blooded notothenioids ($P < 0.05$, Fig. 2.3 C). Similar to SOD1 and SOD2, transcript levels of CAT were not significantly different between the two red-blooded fishes, *N. coriiceps* and *G. gibberifrons* ($P > 0.05$, Fig 2.3 C).

2.4.4 Activity of antioxidants

Similar to transcript levels, the activity of antioxidants were not affected by exposure to CTmax, and tended to be higher in red-blooded fishes compared to icefishes. Maximal activity of SOD and catalase did not change in heart ventricle of the two icefish species or the red-blooded notothenioid *N. coriiceps* in response to exposure to CTmax ($P > 0.05$, Fig 2.4 A, B) but decreased 1.2-times in the heart ventricle of *G. gibberifrons* in response to exposure to CTmax ($P < 0.05$, Fig. 2.4 A). Maximal activity of SOD was 1696.5 ± 66.4 U g wet tissue^{-1} and 1777.1 ± 46.4 U g wet tissue^{-1} in the heart ventricle of the two icefish species *C. aceratus* and C. *rastrospinosus*, respectively, which was significantly lower compared to the red-blooded *G. gibberifrons* (2685.6 ± 51.6 U g wet tissue^{-1}) and *N. coriiceps* (2958.3 ± 100.7 U g wet tissue^{-1}) ($P < 0.05$, Fig. 2.4 A). Maximal activity of catalase was 134.5 ± 14.9 μmol min^{-1} g wet tissue^{-1} in ventricles of *C. aceratus*, which was 2-times lower compared to *C. rastrospinosus* and 2.9-times and 4.0-times lower compared to the two red-blooded species at 0°C ($P < 0.05$, Fig. 2.4 B). Catalase activity was not significantly different between ventricles of the two red-blooded species ($P > 0.05$, Fig. 2.4 B).

Maximal activity of SOD and catalase in pectoral adductor muscle were also not affected by exposure to CTmax in any species ($P > 0.05$, Fig. 2.4 C, D). Similar to heart ventricle, however, maximal activity of SOD was 2.1-times lower in pectoral adductor muscle of the two icefishes, *C. aceratus* and *C. rastrospinosus* compared to the red-blooded species, *N. coriiceps* ($P < 0.05$, Fig. 2.4 C) but was not different between *G. gibberifrons* and *N. coriiceps* ($P > 0.05$, Fig. 2.4 C). Maximal activity of catalase was 1.6-times to 1.7-times lower in pectoral adductor muscle of *C. aceratus* compared to the red-blooded species ($P < 0.05$, Fig. 2.4 D), but not was significantly different between the two icefish species or the two red-blooded species ($P > 0.05$, Fig. 2.4 D).

While maximal activity of SOD was always significantly higher in pectoral adductor muscle compared to heart ventricle in all species ($P < 0.05$, Fig. 2.4 A, C), maximal

activity of catalase did not show a consistent trend between tissues. Maximal activity of catalase was higher in pectoral adductor muscle of *C. aceratus* compared to hearts, yet lower in pectoral adductor muscle of *N. coriiceps* compared to hearts, and similar between hearts and pectoral adductor muscles in *C. rastrospinosus* and *G. gibberifrons* (Fig. 2.4 B, D).

2.5 DISCUSSION

This is the first study to quantify changes in oxidative stress in response to warming in Antarctic notothenioid fishes. We determined that levels of protein carbonyls, a marker for oxidized proteins, and MDA, a marker for peroxidized lipids, increase only in hearts of icefishes, and not in red-blooded notothenioids in response to warming, suggesting that oxidative damage may contribute to the lower thermal tolerance of icefishes compared to red-blooded species. We also determined that despite higher levels of oxidized proteins and lipids in some notothenioids, neither transcript levels nor maximal activity of antioxidants were altered in response to oxidative damage during short-term elevations in temperature.

2.5.1 Hearts of Antarctic icefishes are more susceptible to oxidative damage during warming than red-blooded fishes

The higher levels of oxidized macromolecules in hearts of icefishes compared to red-blooded species may be due to differences in rates of production of ROS and/or levels of antioxidant defenses. Rates of ROS production are similar in mitochondria isolated from heart ventricles of icefishes and red-blooded notothenioids at 2°C and 10°C (Mueller et al., 2011). However, *in vivo*, ROS production may be amplified in oxidative muscles of icefishes by their high volume density of mitochondrial membranes, rich in

polyunsaturated fatty acids. Mitochondrial density is 2.0-times to 2.3-times higher in hearts of *C. aceratus* and 1.1-times to 1.3-times higher in hearts of *C. rastrospinosus* compared to *G. gibberifrons* and *N. coriiceps* (O'Brien and Sidell, 2000; Urschel and O'Brien, 2008). Furthermore, mitochondrial membranes of Antarctic notothenioids have 2.0-times more polyunsaturated fatty acids than cold-acclimated rainbow trout (Kraffe et al., 2007; Mueller et al., 2011). Polyunsaturated fatty acids can undergo lipid peroxidation and once oxidized, can further promote oxidative stress via a self-propagating lipid peroxidation cycle (Cosgrove et al., 1987; Girotti, 1998). This cycle is initiated by hydroxyl radicals, which easily abstract allylic hydrogens from unsaturated lipids, producing a lipid radical. In the presence of molecular oxygen, the lipid radical is converted into lipid hydroperoxide species, which react with additional unsaturated phospholipids, producing more lipid radicals, and perpetuating the peroxidation cycle (reviewed in Girotti, 1998).

Hypoxia may also promote the formation of ROS during warming. Hearts of fishes often become hypoxic as temperature increases because most lack a coronary circulation (Farrell, 2002). Like most teleosts, Antarctic fishes have a type I spongy heart, oxygenated by venous blood (Agnisola and Tota, 1994; Zummo et al., 1995). Hearts of icefishes may be especially vulnerable to hypoxia during warming because of their reduced blood oxygen-carrying capacity compared to red-blooded species. Hypoxia can promote the production of ROS because it prolongs the reduction state of electron carriers within the electron transport chain, particularly at complex III. This increases the likelihood for interactions between ubisemiquinone and molecular oxygen, which produce ROS (Guzy and Schumacker, 2006). The effect of hypoxia can be mimicked *in vitro* by the addition of antimycin A (Guzy and Schumacker, 2006), and previously we determined that rates of ROS production are up to 6-times higher in mitochondria isolated from hearts of icefishes treated with antimycin A compared to untreated ones (Mueller et al., 2011). In addition, ROS production was up 4-times higher in mitochondria of icefishes treated with antimycin A compared to treated mitochondria of

red-blooded fishes (Mueller et al., 2011). Thus, if hearts of notothenioids become hypoxic during warming, icefishes are likely to produce more ROS than red-blooded species.

Icefishes have lower levels of enzymatic antioxidant defenses against ROS compared to red-blooded notothenioids, which may also contribute to oxidative stress during warming. The maximal activity of SOD, the only antioxidant that detoxifies superoxide anions, is up to 1.7-times lower in heart ventricles of the icefishes *C. aceratus* and *C. rastrospinosus* compared to the red-blooded fish species *G. gibberifrons* and *N. coriiceps*. Similarly, the maximal activity of catalase, one of several enzymes that degrade hydrogen peroxide, is up to 4.0-times lower in heart ventricles of *C. aceratus* and tended to be lower in *C. rastrospinosus* compared to the two red-blooded notothenioids. These findings are in agreement with a previous study that determined levels of SOD are 6.0-times and catalase 4.0-times lower in heart ventricles of the icefish *C. hamatus* compared to the red-blooded notothenioid *T. bernacchii* (Cassini et al., 1993).

Surprisingly, levels of oxidized macromolecules did not increase in pectoral adductor muscle of any species during warming. This may be due to differences in antioxidant levels and/or rates of ROS production between hearts and pectoral muscles. The maximal activity of SOD is 1.4-times higher in the pectoral adductor muscle of *C. aceratus* and 1.3-times higher in *C. rastrospinosus* compared to heart ventricles. Additionally, maximal activity of catalase was 1.4-times higher in pectoral adductor muscle of *C. aceratus* compared to the heart ventricle. Typically, levels of antioxidant defenses are correlated with aerobic metabolic capacity in fishes (Crockett, 2011) but this trend is not apparent in hearts and pectoral muscles of icefishes. In fact, maximal activity of cytochrome *c* oxidase per gram of tissues is lower in pectoral muscle of the icefishes *C. aceratus* and *C. rastrospinosus* compared to heart ventricle, while SOD and catalase activity are higher (O'Brien and Sidell, 2000; O'Brien et al., 2003), suggesting

antioxidants may be protecting against sources of ROS in addition to those produced by the respiratory chain in pectoral adductor muscles of icefishes.

Rates of ROS production have only been measured in mitochondria isolated from the heart ventricle and not in oxidative pectoral adductor muscle of Antarctic notothenioids so we do not know if rates of ROS production are lower in pectoral muscle compared to hearts (Mueller et al., 2011). However, if hypoxia contributes to ROS production, the pectoral adductor muscle is likely to be less affected than hearts. In contrast to the heart, which is supplied by venous blood, the pectoral adductor muscle is supplied with freshly-oxygenated blood via a hypobranchial shunt, branching directly from the first three gill arches (Egginton and Rankin, 1998).

2.5.2 Antarctic notothenioids do not up-regulate their antioxidant defenses in response to warming

Despite an increase in oxidized proteins and/or lipids in hearts of the icefishes *C. aceratus* and *C. rastrospinosus* during warming, neither species increased mRNA levels or activities of antioxidants. Antarctic notothenioids are amongst the most stenothermic animals on Earth and a high degree of stenothermy is often accompanied by loss of thermal plasticity. The best described example of this is the loss of the heat shock response in notothenioids (reviewed in Feder and Hofmann, 1999). While notothenioids constitutively express heat shock proteins (Hsps), neither the expression nor activity of Hsps is elevated in response to heat shock (Carpenter and Hofmann, 2000; Hofmann et al., 2000; Hofmann and Place, 2005). Moreover, notothenioids have a blunted response to thermal stress compared to temperate fishes. The expression of 9502 genes was analyzed in gills of *T. bernacchii* exposed to 4°C for 4 hours, and transcript levels of only 262 genes changed in response to heat shock, none of which were associated with the antioxidant defense (Buckley and Somero, 2009). In contrast, SOD activity increased up to 3.0-times in liver and up to 4.0-times in brain and kidney of eurythermal goldfish

(*Carassius auratus*) after being exposed to an increase in temperature from 21°C to 35°C for 6-12 hours (Lushchak and Bagnyukova, 2006). These results and those from the current study suggest that similar to the Hsp response, notothenioids may have lost the capacity to regulate transcript levels and activities of antioxidants in response to warming. However, we cannot rule out the possibility that antioxidant levels might increase in response to prolonged exposure to elevated temperature and future studies will address this question.

2.5.3 Inter-relationship between the expression of oxygen-binding proteins, oxidized macromolecules and antioxidants

There is a positive correlation between the expression of oxygen-binding proteins, levels of oxidized proteins, and levels of antioxidants among notothenioids, with levels of oxidized proteins and antioxidants tending to be highest in fishes expressing the oxygen-binding proteins Hb and myoglobin (Mb). Mb is expressed in the heart ventricle of the two red-blooded species and the icefish *C. rastrospinosus* but is absent in the heart ventricle of the icefish *C. aceratus* (Sidell et al., 1997; Vayda et al., 1997; Moylan and Sidell, 2000). Hb and Mb, like other heme-containing proteins, exhibit peroxidase activity. Oxidation of a ferrous heme protein by superoxide radicals or hydrogen peroxide produces ferryl Mb/Hb and protein-based radicals, which are powerful oxidants capable of damaging proteins and lipids (reviewed in Reeder and Wilson, 2005). In addition, when Fe^{2+} is released from damaged heme proteins, it participates in the Fenton reaction, producing highly reactive hydroxyl radicals from hydrogen peroxide, which not only damage macromolecules, but also initiate the lipid peroxidation cycle (reviewed in Girotti, 1998). The higher levels of antioxidants in red-blooded notothenioids compared to icefishes may be necessary to counter the oxidative reactions originating from the heme-based proteins Hb and Mb. Higher rates of protein synthesis in red-blooded species are consistent with higher rates of damage because oxidatively-damaged proteins are degraded by 20S proteasome complexes and must be replaced with newly-synthesized

ones (reviewed in Davies, 2001). Previous studies have shown that rates of ^{14}C-phenylalanine incorporation are 3.1-times lower in heart ventricles and 4.3-times lower in pectoral muscles of *C. aceratus* compared to *N. coriiceps* at 2°C (Haschemeyer, 1983).

The loss of Hb and Mb is considered a "disaptation"- a neutral mutation that has persisted in the population because it does not negatively impact fitness (reviewed in Sidell and O'Brien, 2006). However, if the presence of Hb and Mb promotes oxidative damage, elevates rates of protein synthesis, and warrants higher levels of antioxidants, then the loss of these oxygen-binding proteins may offer an energetic advantage. Protein synthesis is an energetically costly process, with approximately 15-22% of an organism's standard metabolic rate being allocated to the synthesis of new proteins (Rolfe and Brown, 1997). Future studies will address the question of whether the loss of expression of oxygen-binding proteins might protect against damaging oxidative reactions.

2.5.4 Conclusion

Our study suggests increases in levels of oxidized macromolecules, which only occurred in hearts of icefishes, might contribute to the reduced thermal tolerance of icefishes. However, we cannot rule out the possibility that the lower CTmax of icefishes may be due to their reduced oxygen-carrying capacity compared to red-blooded species. Indeed, there is a significant positive correlation between hematocrit and CTmax amongst notothenioids (Beers and Sidell, 2011). Future studies will empirically test this hypothesis.

Although levels of oxidized macromolecules increased in hearts of icefishes in response to warming, absolute levels of oxidized proteins were always higher in the heart ventricle of the red-blooded *N. coriiceps* compared to the two icefishes. The presence of oxidized macromolecules is not harmful *per se*. Controlled protein and lipid oxidation, balanced by antioxidants, contributes to the oxidant-antioxidant balance, or redox tone

(reviewed in Droge, 2002; Crockett, 2008), which differs among tissues and species. Short term changes in redox status due to minor elevations in ROS production regulate signaling pathways and the activity of signaling molecules including, MAPK, NFκB, protein tyrosine phosphatases, Src family kinases and JNK kinases (reviewed in Droge, 2002). However, prolonged, extreme shifts in redox tone damage macromolecules, causing oxidative stress (reviewed in Droge, 2002). Thus, higher levels of oxidized macromolecules in muscles of red-blooded notothenioids might not be deleterious. However, the temperature-induced increases in levels of oxidized proteins and lipids in icefishes might shift the balance of redox status and impair function of specific proteins and lipids, thereby reducing thermal tolerance.

2.6 ACKNOWLEDGMENTS

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2.8 FIGURES

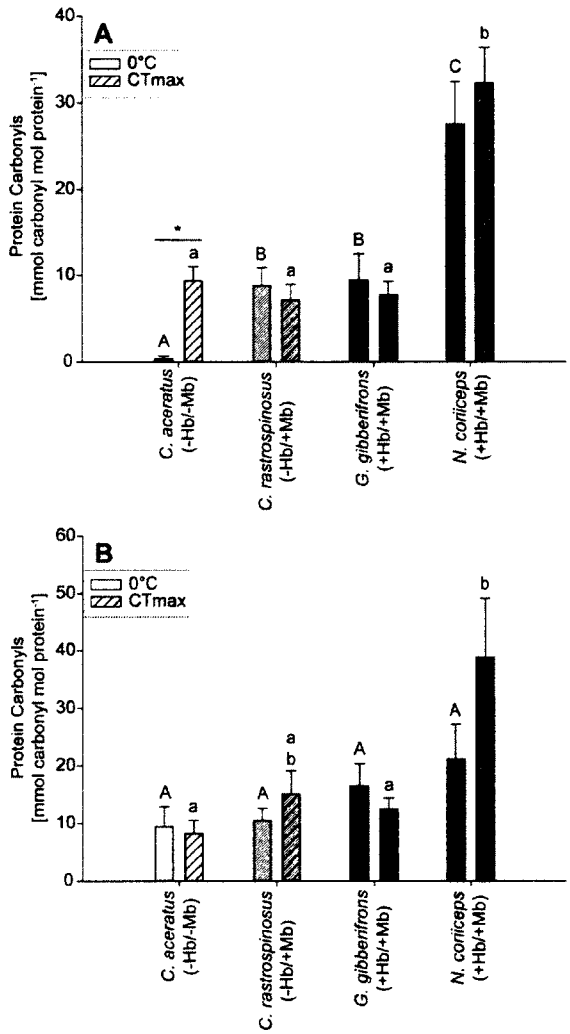

Figure 2.1 Levels of oxidized proteins in Antarctic notothenioid fishes

Levels of oxidized proteins [mmol carbonyls mol protein^{-1}] in ventricle (A) and pectoral adductor muscle (B) of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps*. Animals were either held at 0°C (solid bars) or exposed to their respective CTmax (hatched bars). N = 7–11. Differences between species at a common temperature are indicated by different capital (0°C) or small (CTmax) letters. Significant differences between temperature treatments within a species are indicated by asterisks. $P < 0.05$.

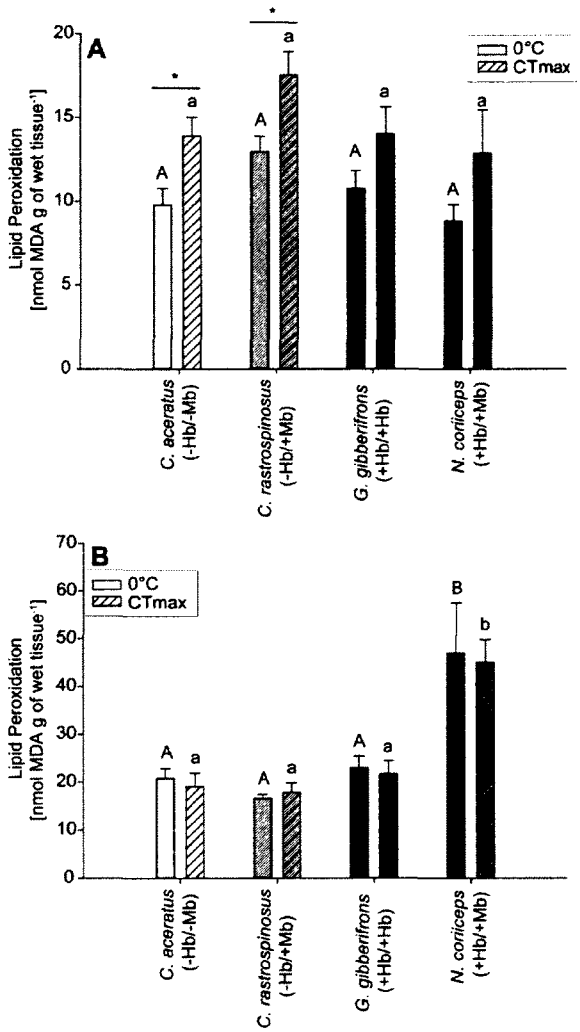

Figure 2.2 Levels of oxidized phospholipids in Antarctic notothenioid fishes

Levels of oxidized phospholipids [nmol MDA g wet tissue^{-1}] in ventricle (A) and pectoral adductor muscle (B) of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps*. Animals were either held at 0°C (solid bars) or exposed to their respective CTmax (hatched bars). N = 6–14. Differences between species at a common temperature are indicated by different capital (0°C) or small (CTmax) letters. Significant differences between temperature treatments within a species are indicated by asterisks. $P < 0.05$.

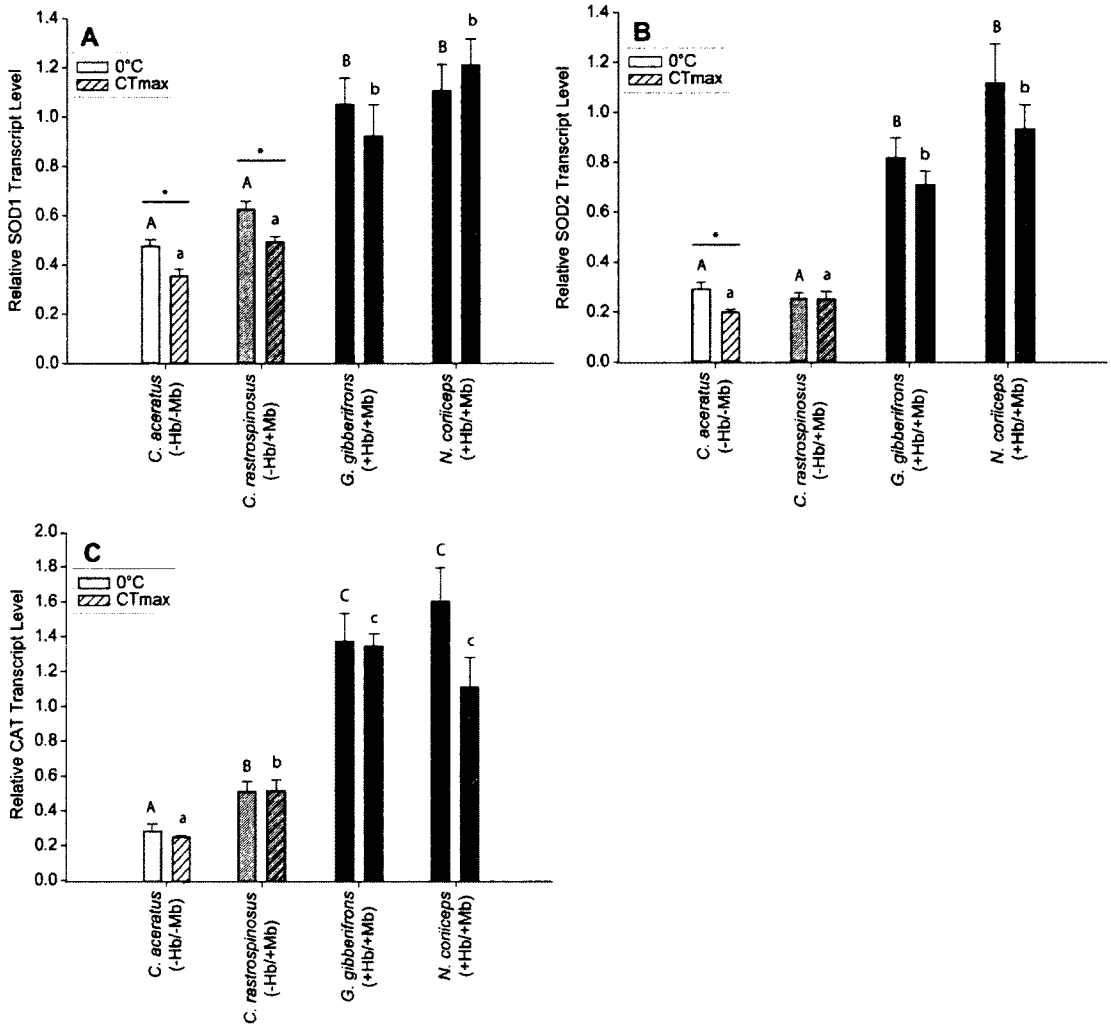

Figure 2.3 Antioxidant transcript levels in Antarctic notothenioid fishes

Relative transcript levels of SOD1 (A) and SOD2 (B) and CAT (C) in ventricle of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps*. Animals were either held at 0°C (solid bars) or exposed to their respective CTmax (hatched bars). Transcript levels of SOD1, SOD2 and CAT were normalized to transcript levels of EF-1α. N = 6–8. Differences between species at a common temperature are indicated by different capital (0°C) or small (CTmax) letters. Significant differences between temperature treatments within a species are indicated by asterisks. $P < 0.05$.

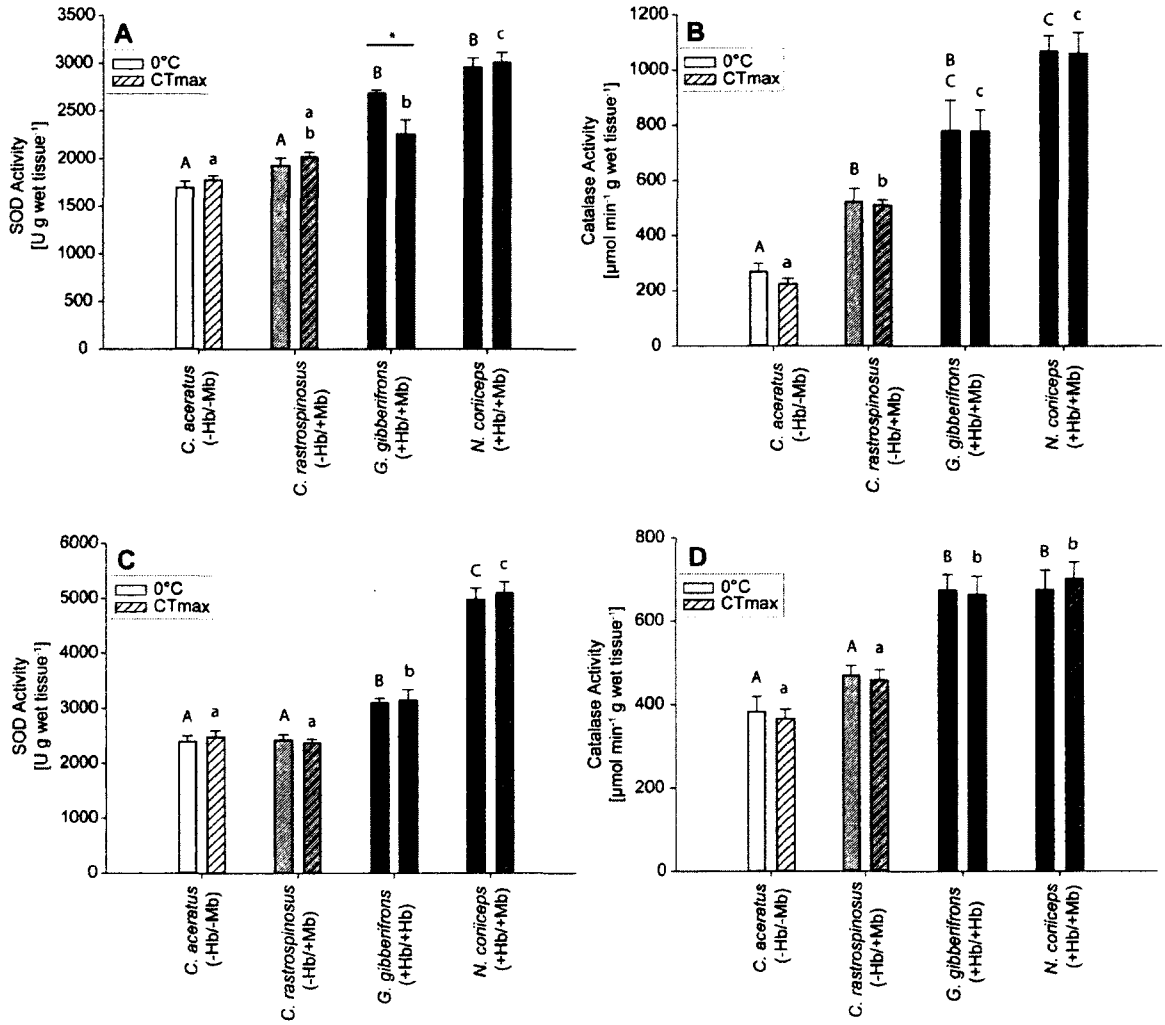

Figure 2.4 Antioxidant activities in Antarctic notothenioid fishes

Activity of SOD [Units g wet tissue^{-1}] (A, C) and catalase [μmol min^{-1} g wet tissue^{-1}] (B, D) in ventricle (A, B) and pectoral adductor muscle (C, D) of *C. aceratus*, *C. rastrospinosus*, *G. gibberifrons* and *N. coriiceps*. Animals were either held at 0°C (solid bars) or exposed to their respective CTmax (hatched bars). N = 6–8. Differences between species at a common temperature are indicated by different capital (0°C) or small (CTmax) letters. Significant differences between temperature treatments within a species are indicated by asterisks. $P < 0.05$

2.9 TABLES

Table 2.1 Degenerate and gene-specific primers

Degenerate and gene-specific primers used for quantification of SOD1, SOD2, CAT and EF-1α transcript levels. Degenerate and gene-specific primers for EF-1α were published previously (Urschel and O'Brien, 2008).

Gene	Type	Primer	Amplicon
SOD1	*degenerate*	F 5' CGTGCACGCCTTCGGNGAYAAYAC 3'	291 bp
		R 5' GGCCGCCGGCGTTNCCNGTYTT 3'	
SOD1	*specific*	F 5' CAAACGGGTGCATCAGTGC 3'	101 bp
		R 5' CACATTCCCCAGGTCTCCAA 3'	
SOD2	*degenerate*	F 5' GCAGCTGCACCACTCCAARCAYCAYGC 3'	300 bp
		R 5' TCCTTGTTGTATCCCAGCCANCCCCANCC 3'	
SOD2	*specific*	F 5' GAGGAGAGCCACAGGGGG 3'	60 bp
		R 5' TCTGGAAGGAGCCAAAGTCCC 3'	
CAT	*degenerate*	F 5' GAAGTTCTACACCGAGGAGGGNAAYTGGGA 3'	798 bp
		R 5' GCCCTGGTTGTCGTGCATRCACATNGG 3'	

Table 2.1 Degenerate and gene-specific primer continued

Gene	Type	Primer	Amplicon
CAT	*specific*	F 5' CATGAAAGACCCCGACATGG 3'	82 bp
		R 5' GTCGCTGAACAAGAAAGACACCT 3'	
EF-1α	*degenerate*	F 5' CGACATCGCCCTGTGGAARTTYGARAC	561 bp
		R 5' GATGGCCGCCGATCTTGTANACRTCYTG	
EF-1α	*specific*	F 5' CTGGAAGCCAGTGAAAAGATGAC 3'	51 bp
		R 5' ACGCTCAACCTTCCATCCC 3'	

Degenerate nucleotides are indicated by N, R and Y (N = A or C or G or T; R = A or G; Y = C or T)

2.10 REFERENCES

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2.11 APPENDICES

C GTG CAC GCC TTC GGG GAT AAT ACA AAC GGG TGC ATC AGT GCA GGC CCT CAC TTC AAT CCC CAC AAC AAG ACT CAC GCC GGT CCT ACT GAT GAA AAT AGG CAT GTT GGA GAC CTG GGG AAT GTG ACT GCT GCA GCT GAT AAT GTT GCA AAG CTC GAC ATC ACG GAC AAG ATG ATC ACC CTT GCT GGC CAA TAC TCT ATT ATT GGC AGA ACC ATG GTG ATC TAT GAG AAG GCC GAC GAC CTG GGA AAA GGA GGC AAT GAG GAG AGT CTA AAG ACA GGC AAC GCC GGC GGC CA

Appendix 2.1 Partial mRNA sequence of SOD1 in *C. aceratus*

Partial sequence was obtained using degenerate primers shown in Table 2.1. Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

C GTG CAC GCC TTC GGG GAT AAC ACA AAC **GGG** TGC ATC AGT GCA GGC CCT CAC TTC AAT CCC CAC AAC AAG ACT CAT GCC GGT CCT ACT GAT GAA AAT AGG CAT GTT GGA GAC CTG GGG AAT GTG ACT GCT GCA GCT GAT AAT GTT GCA AAG CTC GAC ATC ACG GAC AAG ATG ATC ACC CTT GCT GGC CAA TAC TCT ATT ATT GGC AGA ACC ATG GTG ATC CAT GAG AAG GCC GAC GAC CTG GGA AAA GGA GGC AAT GAG GAG AGT CTA AAG ACG GGC AAC GCC GGC GGC CA

Appendix 2.2 Partial mRNA sequence of SOD1 in *C. rastrospinosus*

Partial sequence was obtained using degenerate primers shown in Table 2.1. Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

C GTG CAC GCC TTC GGG GAT AAC ACA AAC **GG**G TGC ATC AGT GCA GGC CCT CAC TTC AAT CCC CGC AAC AAG ACT CAT GCC GGT CCT ACT GGT GAA AAT AGG CAT GTT GGA GAC CTG GGG AAT GTG ACT GCT GCA GCT GAT AAT GTT GCA AAG CTC GAC ATC ACG GAC AAG ATG ATC ACT CTT GCT GGC CAA TAC TCT ATT ATT GGC AGA ACC ATG GTG ATC CAT GAG AAG GCC GAC GAC CTG GGA AAA GGA GGC AAT GAT GAG AGT CTA AAG ACC GGT AAC GCC GGC GGC CA

Appendix 2.3 Partial mRNA sequence of SOD1 in *G. gibberifrons*

Partial sequence was obtained using degenerate primers shown in Table 2.1. Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

C GTG CAC GCC TTC GGG GAT AAT ACA AAC **GG**G TGC ATC AGT GCA GGC CCT CAC TTC AAT CCC CAC AAC AAG ACT CAT GCC GGT CCT ACT GAT GAA AAT GGG CAT GTT GGA GAC CTG GGG AAT GTG ACT GCT GCA GCT GAT AAT GTT GCA AAG CTC GAC ATC ACG GAC AAG ATG ATC ACC CTT GCT GGC CAA TAC CCT ATT ATT GGC AGA ACC ATG GTG ATC CAT GAG AGG GCC GAC GAC CTG GGA AAA GGA GGC AAT GAT GAG AGT CTA AAG ACG GGA AAC GCC GGC GGC CA

Appendix 2.4 Partial mRNA sequence of SOD1 in *N. coriiceps*

Partial sequence was obtained using degenerate primers shown in Table 2.1. Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

G CAG CTG CAC CAC TCC AAG CAT CAC GCC ACA TAC GTG AAC AAC CTC AAT GTT ACA GAG GAG AAA TAT CAG GAG GCA CTA GCA AAG GGA GAT GTG ACT GCA CAG GTG GCC CTC CAG CCC GCT CTG AAG TTT AAT GGA GGC GGC CAC ATT AAC CAC TCT ATC TTC TGG ACA AAC CTC TCT CCC AAT GGG GGA GGA GAG CCA CAG GGG GAG CTG ATG GAG GCC ATT AAG CGG GAC TTT GGC TCC TTC CAG AAG GTG AAG GAG AGG ATG TCT GCG GCG ACA GTT GCA GTG CAG GGG TCA GGG TGG GGC TGG CTG GGA TAC A

Appendix 2.5 Partial mRNA sequence of SOD2 in *C. aceratus*

Partial sequence was obtained using degenerate primers shown in Table 2.1. Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

G CAG CTG CAC CAC TCC AAG CAT CAC GCC ACA TAT GTG AAC AAC CTC AAT GTT ACA GAG GAG AAA TAT CAG GAG GCA CTA GCA AAG GGA GAT GTG ACG GCA CAG GTG GCC CTC CAG CCC GCT CTG AGG TTT AAT GGA GGC GGC CAC ATT AAC CAC TCT ATC TTC TGG ACA AAC CTC TCT CCT AAT GGG GGA GGA GAG CCA CAG GGG GAG CTG ATG GAG GCC ATT AAG CGG GAC TTT GGC TCC TTC CAG AAG ATG AAG GAG AGG ATG TCT GCG TCG ACA GTT GCA GTG CAG GGG TCA GGG TGG GGC TGG CTG GGA TAC A

Appendix 2.6 Partial mRNA sequence of SOD2 in *C. rastrospinosus*

Partial sequence was obtained using degenerate primers shown in Table 2.1. Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

G CAG CTG CAC CAC TCC AAG CAT CAC GCC ACA TAT GTG AAC AAC CTC AAT GTT ACA GAG GAG AAA TAT CAG GAG GCA CTA GCA AAG GGA GAT GTG ACG GCA CAG GTG GCC CTC CAG CCC GCT CTG AAG TTT AAT GGA GGC GGC CAC ATT AAC CAC TCA ATC TTC TGG ACA AAC CTC TCT CCT AAT GGG GGA GGA GAG CCA CAG GGG GAG CTG ATG GAG GCC ATT AAG CGG GAC TTT GGC TCC TTC CAG AGG ATG AAG GAG AGG ATG TCG GCG GCA ACA GTG GCC GTG CAG GGG TCA GGT TGG GGC TGG CTG GGA TAC A

Appendix 2.7 Partial mRNA sequence of SOD2 in *G. gibberifrons*

Partial sequence was obtained using degenerate primers shown in Table 2.1. Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

G CAG CTG CAC CAC TCC AAG CAT CAT GCC ACA TAT GTG AAC AAC CTC AAT GTT ACA GAG GAG AAA TAT CAG GAG GCA CTA GCA AAG GGA GAT GTG ACG GCA CAG GTG GCC CTC CAG CCC GCT CTG AAG TTT AAT GGA GGT GGC CAC ATT AAC CAC TCT ATC TTC TGG ACA AAC CTC TCT CCT AAT GGG GGA GGA GAG CCA CAG GGG GAG CTG ATG GAG ACC ATT AAG CGG GAC TTT GGC TCC TTC CAG AAG ATG AAG GAG AGG ATG TCT GCG GCG ACA GTG GCC GTG CAG GGG TCA GGT TGG GGC TGG CTG GGA TAC A

Appendix 2.8 Partial mRNA sequence of SOD2 in *N. coriiceps*

Partial sequence was obtained using degenerate primers shown in Table 2.1. Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

G AAG TTC TAC ACC GAG GAG GGG AAC TGG GAC CTG ACG GGC AAC AAC ACC CCC ATC TTC TTC ATC AGG GAC GCC CTG CTG TTC CCG TCC TTC ATC CAT TCC CAG AAG CGC AAT CCC CAG ACC CAC ATG AAA GAC CCC GAC ATG GTG TGG GAC TTC TGG AGC CTG AGG CCT GAG AGT CTG CAT CAG GTG TCT TTC TTG TTC AGC GAC CGA GGT TTG CCT GAT GGT TTC CGT CAT ATG AAC GGC TAC GGC TCA CAC ACT TTC AAA ATG GTC AAT ACC CAA GGA GAG CCT TTC TAC TGC AAG TTC CAC TTC AAG ACT GAT CAA GGA ATA AGG AAC ATG TCA GGG GAG GAG GCA GAG CGC CTG GCT GCC AGC AAC CCA GAT TAT GCC ATT GGA GAT CTT TAC AAC GCC ATT GCT AAT GGA AAC TTC CCA TCC TGG ACC TTC TTC ATC CAG ATC ATG ACC TTC GAG CAG GCG GAG ACC TTC CGG TTC AAC CCC TTC GAT CTC ACC AAG GTT TGG TCT CAA AAA GAA TAC CCT TTG ATC CCT GTG GGC AAA ATG GTC CTC AAC AGG AAT GCA GTG AAC TAC TTT GCC GAG ATA GAG CAG CTG GCC TTT GAC CCC AGC AAC ATG CCG CCT GGC ATC GAG GCG AGC CCC GAC AAG ATG CTG CAG GGT CGT CTC TTC TCT TAC CCA GAC ACA CAT CGA CAC CGG CTG GGA GCA AAC TAC CTG CAG CTC CCC GTC AAC TGT CCC TTC AGG ACC CGC GTG ACC AAC TAC CAG CGC GAT GGC C

Appendix 2.9 Partial mRNA sequence of CAT in *C. aceratus*

Partial sequence was obtained using degenerate primers shown in Table 2.1. Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

G AAG TTC TAC ACC GAG GAG GGT AAC TGG GAC CTG ACG GGC AAC AAC ACC CCC ATC TTC TTC ATC AGG GAC GCC CTG CTG TTC CCG TCC TTC ATC CAT TCC CAG AAG CGC AAT CCC CAG ACC CAC ATG AAA GAC CCC GAC ATG GTG TGG GAC TTC TGG AGC CTG AGG CCT GAG AGT CTG CAT CAG GTG TCT TTC TTG TTC AGC GAC CGA GGT TTG CCT GAT GGT TTC CGT CAT ATG AAC GGC TAC GGC TCA CAC ACT TTC AAA ATG GTC AAT ACC CAA GGA GAG CCT TTC TAC TGC AAG TTC CAC TTC AAG ACT GAT CAA GGA ATA AAG AAC ATG TCA GGG GAG GAG GCA GAG CGC CTA GCT GCC AGC AAC CCA GAT TAT GCC ATT GGA GAT CTT TAC AAC GCC ATT GCT AAT GGA AAC TTC CCA TCC TGG ACC TTC TTC ATC CAG ATC ATG ACC TTC GAG CAG GCG GAG ACC TTC CAG TTC AAC CCC TTC GAT CTC ACC AAG GTT TGG TCT CAA AAA GAA TAC CCT TTG ATC CCT GTG GGC AAA ATG GTC CTC AAC AGG AAT GCA TTG AAC TAC TTT GCC GAG ATA GAG CAG CTG GCC TTT GAC CCC AGC AAC ATG CCG CCT GGC ATC GAG GCG AGC CCC GAC AAG ATG CTG CAG GGT CGT CTC TTC TCT TAC CCA GAT ACA CAT CGA CAC CGG CTG GGA GCA AAC TAC CTG CAG CTC CCC GTC AAC TGT CCT TTC AGG ACC CGC GTG ACC AAC TAC CAG CGC GAT GGC C

Appendix 2.10 Partial mRNA sequence of CAT in *C. rastrospinosus*

Partial sequence was obtained using degenerate primers shown in Table 2.1. Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

G AAG TTC TAC ACC GAG GAG GGG AAC TGG GAC CTG ACG GGC AAC AAC ACC CCC ATC TTC TTC ATC AGG GAC GCC CTG CTG TTC CCG TCC TTC ATC CAT TCC CAG AAG CGC AAT CCC CAG ACC CAC ATG AAA GAC CCC GAC ATG GTG TGG GAC TTC TGG AGC CTG AGG CCT GAG AGT CTG CAT CAG GTG TCT TTC TTG TTC AGC GAC CGA GGT TTG CCT GAT GGT TTC CGT CAT ATG AAC GGC TAC GGC TCA CAC ACT TTC AAA ATG GTC AAT ACC CAA GGA GAG CCT TTC TAC TGC AAG TTC CAC TTC AAG ACT GAT CAA GGA ATA AAG AAC ATG TCA GGG GAA GAG GCA GAG CGC CTG GCT GCC AGC AAC CCA GAT TAT GCC ATT GGA GAT CTT TAC AAC GCC ATT GCT AAT GGA AAC TTC CCA TCC TGG ACC TTC TTC ATC CAG ATC ATG ACC TTC GAG CAG GCG GAG ACC TCC CAA TTG CCC TCC T

Appendix 2.11 Partial mRNA sequence of CAT in *G. gibberifrons*

Partial sequence was obtained using degenerate primers shown in Table 2.1. Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

G AAG TTC TAC ACC GAG GAG GGG AAT TGG GAC CTG ACG GGC AAC AAC ACC CCC ATC TTC TTC ATC AGG GAC GCC CTG CTG TTC CCG TCC TTC ATC CAT TCC CAG AAG CGC AAT CCC CAG ACC CAC ATG AAA GAC CCC GAC ATG GTG TGG GAC TCC TGG AGC CTG AGG CCT GAG AGT CTG CAT CAG GTG TCT TTC TTG TTC AGC GAC CGA GGT TTG CCT GAT GGT TTC CGT CAT ATG AAC GGC TAC GGC TCA CAC ACT TTC AAA ATG GTC AAT ACC CAA GGA GAG CCT TTC TAC TGC AAG TTC CAC TTC AAG ACT GAT CAA GGA ATA AAG AAC ATG TCA GGG GAG GAG GCA GAG CGC CTA GCT GCC AGC AAC CCA GAT TAT GCC ATT GGA GAT CTT TAC AAC GCC ATT GCT AAT GGA AAC TTC CCA TCC TGG ACC TTC TTC ATC CAG ATC ATG ACC TTC GAG CAG GCG GAG ACC TTC CAG TTC AAC CCC TTC GAT CTC ACC AAG GTT TGG TCT CAA AAA GAA TAC CCT TTG ATC CCT GTG GGC AAA ATG GTC CTC AAC AGG AAT GCA TTG AAC TAC TTT GCC GAG ATA GAG CAG CTG GCC TTT GAC CCC AGC AAC ATG CCG CCT GGC ATC GAG GCG AGC CCC GAC AAG ATG CTG CAG GGT CGT CTC TTC TCT TAC CCA GAT ACA CAT CGA CAC CGG CTG GGA GCA AAC TAC CTG CAG CTC CCC GTC AAC TGT CCT TTC AGG ACC CGC GTG ACC AAC TAC CAG CGC GAT GGC C

Appendix 2.12 Partial mRNA sequence of CAT in *N. coriiceps*

Partial sequence was obtained using degenerate primers shown in Table 2.1. Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

CG ACA TCG CCC TGT GGA AGT TTG AGA CTG CCA AGT ACT ACG TGA CCA TCA TTG ATG CCC CTG GAC ACA GGG ATT TCA TCA AGA ACA TGA TCA CTG GTA CCT CTC AGG CTG ACT GCG CTG TGC TGA TCG TTG CTG CCG GTG TTG GTG AGT TTG AGG CCG GTA TCT CTA AGA ACG GCC AGA CCC GTG AGC ACG CCC TGC TGG CTT TCA CCC TCG GTG TGA AGC AGC TCA TCG TAG GAG TCA ACA AGA TGG ACT CCA CCG AGC CCC CTT ACA GCC AAG CCC GTT ATG AAG AAA TCG CCA AGG AAG TGA GCA CTT ACA TCA AGA AGA TCG GCT ACA ACC CCT TAA CTG TGC CCT TTG TCC CCA TCT CTG GAT GGC ACG GAG ACA ACA TGC TGG AAG CCA GTG AAA AGA TGA CAT GGT TCA AGG GAT GGA AGG TTG AGC GTA AGG AGG GTA ATG CCA GTG GAG TCA CCC TGC TGG AAT CTC TCG ATG CCA TCC TGC CCC CGT CCC GCC TCA CCG ACA AGC CCC TCC GTC TGC CCC TGC AGG ACG TCT ACA AGA TCG GCG GCC AT

Appendix 2.13 Partial mRNA sequence of EF-1α in *C. aceratus*

Partial sequence was previously published by Urschel and O'Brien, 2008 (GenBank: EU857824). Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

CG ACA TCG CCC TGT GGA AGT TCG AAA CTG CCA AGT ACT ACG TGA CCA TCA TTG ATG CCC CTG GAC ACA GGG ATT TCA TCA AGA ACA TGA TCA CTG GTA CCT CTC AGG CTG ACT GCG CTG TGC TGA TCG TTG CTG CCG GTG TTG GTG AGT TTG AGG CCG GTA TCT CCA AGA ACG GCC ACA CCC GTG AGC ACG CCC TGC TGG CTT TCA CCC TCG GTG TGA AGC AGC TCA TCG TAG GAG TCA ACA AGA TGG ACT CCA CCG AGC CCC CTT ACA GCC AAG CCC GTT ATG AAG AAA TCG CCA AGG AAG TGA GCA CTT ACA TCA AGA AGA TCG GCT ACA ACC CCT TAA CTG TGC CCT TTG TCC CCA TCT CTG GAT GGC ACG GAG ACA ACA TGC TGG AAG CCA GTG AAA AGA TGA CAT GGT TCA AGG GAT GGA AGG TTG AGC GTA AGG AGG GTA ATG CCA GTG GAG TCA CTC TGC TGG AAT CTC TCG ATG CCA TCC TGC CCC CGT CCC GCC CCA CCG ACA AGC CCC TCC GTC TGC CCC TGC AGG ACG TCT ACA AGA TCG GCG GCC AT

Appendix 2.14 Partial mRNA sequence of EF-1α in *C. rastrospinosus*

Partial sequence was previously published by Urschel and O'Brien, 2008 (GenBank: EU857826). Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

CG ACA TCG CCC TGT GGA AGT TCG AAA CTG CCA AGT ACT ACG TGA CCA TCA TTG ATG CCC CTG GAC ACA GAG ATT TCA TCA AGA ACA TGA TCA CTG GTA CCT CCC AGG CTG ACT GCG CTG TGC TGA TCG TTG CTG CCG GTG TTG GTG AGT TTG AGG CTG GTA TCT CCA AGA ACG GCC AGA CCC GTG AGC ACG CCC TGC TGG CTT TCA CCC TCG GTG TGA AGC AGC TCA TCG TAG GAG TCA ACA AGA TGG ACT CCA CCG AGC CCC CTT ACA GCC AGG CCC GTT ATG AAG AAA TCG CCA AGG AAG TGA GCA CTT ACA TCA AGA AGA TCG GCT ACA ACC CCT TAA CTG TGC CCT TCG TCC CCA TCT CTG GAT GGC ACG GAG ACA ACA TGC TGG AAG CCA GTG AAA AGA TGA CAT GGT TCA AGG GAT GGA AGG TTG AGC GTA AGG AGG GTA ATG CCA ATG GAG TCA CTC TGC TGG AGT CTC TCG ATG CCA TCC TGC CCC CGT CCC GCC CCA CCG ACA AGC CCC TCC GTC TGC CCC TGC AGG ACG TGT ACA AGA TCG GCG GCC AT

Appendix 2.15 Partial mRNA sequence of EF-1α in *G. gibberifrons*

Partial sequence was obtained using degenerate primers shown in Table 2.1. Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

CG ACA TCG CCC TGT GGA AGT TTG AGA CTG CCA AGT ACT ACG TGA CCA TCA TTG ATG CCC CTG GAC ACA GGG ATT TCA TCA AGA ACA TGA TCA CTG GTA CCT CTC AGG CTG ACT GCG CTG TGC TGA TCG TTG CTG CTG GTG TTG GTG AGT TTG AGG CCG GTA TCT CCA AGA ACG GCC AGA CCC GTG AGC ATG CCC TGC TGG CTT TCA CCC TCG GTG TGA AGC AGC TCA TCG TAG GAG TCA ATA AGA TGG ACT CCA CCG AGC CCC CTT ACA GCC AGG CCC GTT ATG AAG AAA TCG CCA AGG AAG TGA GCA CTT ACA TCA AGA AGA TCG GCT ACA ACC CCT TAA CTG TGC CCT TTG TCC CCA TCT CTG GAT GGC ACG GAG ACA ACA TGC TGG AAG CCA GTG AAA **AG**A TGA CAT GGT TCA AGG GAT GGA AGG TTG AGC GTA AGG AGG GTA ATG CCA GTG GAG TCA CTC TGC TGG AGT CTC TCG ATG CCA TCC TCC CCC CGT CCC GCC CCA CCG ACA AGC CCC TCC GTC TGC CCC TGC AAG ACG TCT ACA AGA TCG GCG GCC AT

Appendix 2.16 Partial mRNA sequence of EF-1α in *N. coriiceps*

Partial sequence was previously published by Urschel and O'Brien, 2008 (GenBank: EU857825). Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR. Putative splice sites within annealing sites of the specific primer are indicated with bold letters.

CHAPTER 3: NEITHER LEVELS OF OXIDIZED PROTEINS NOR ANTIOXIDANTS INCREASE IN OXIDATIVE MUSCLE OF WHITE- AND RED-BLOODED ANTARCTIC NOTOTHENIOID FISHES IN RESPONSE TO EXPOSURE TO 4°C[1]

[1] Mueller, I.A. and O'Brien, K. M. 201X. Neither levels of oxidized proteins nor antioxidants increase in oxidative muscle of white- and red-blooded Antarctic notothenioid fishes in response to exposure to 4°C. Prepared for submission to *Polar Biology*

3.1 SUMMARY

Antarctic icefishes have a lower thermal tolerance than closely-related red-blooded notothenioid fishes. Previous studies in our laboratory suggest that oxidative stress might contribute to the lower thermal tolerance of icefishes. Levels of oxidized proteins and/or lipids increased in heart ventricle of icefishes but not red-blooded fishes in response to exposure to their critical thermal maximum (CTmax). In addition, neither icefishes nor red-blooded notothenioids up-regulated their antioxidant defenses in response to CTmax exposure, suggesting that this ability might have been lost in notothenioids during their evolution at cold temperature. These findings raise the question of whether oxidative stress might occur in icefishes in response to a longer exposure to elevated temperature. To address this question, the icefish *Chionodraco rastrospinosus* and the red-blooded fish *Notothenia coriiceps* were maintained at either 0°C or 4°C for one week. Levels of oxidized proteins as well as mRNA and enzyme levels of the antioxidants superoxide dismutase and catalase were quantified in heart ventricle and pectoral adductor muscle of both species. Although previous studies showed that the production of reactive oxygen species in mitochondria isolated from hearts of notothenioids increases with temperature, neither levels of oxidized proteins nor levels of antioxidants increased in tissues of either species in response to exposure to 4°C. This suggests that oxidative stress might not occur in Antarctic fishes in response to prolonged warming.

3.2 INTRODUCTION

The thermal tolerance of Antarctic icefishes is significantly lower than that of closely-related red-blooded Antarctic notothenioid fish species (Beers and Sidell 2011). The critical thermal maximum (CTmax) of the icefishes *Chaenocephalus aceratus* and

Chionodraco rastrospinosus is only 13.9°C and 13.3°C, respectively, whereas the CTmax is 15.5°C in the red-blooded *Gobionotothen gibberifrons* and 17.1°C in the red-blooded *Notothenia coriiceps* (Beers and Sidell 2011). The lower thermal tolerance of icefishes might be due to the low oxygen-carrying capacity of their blood (Beers and Sidell 2011). Oxygen is only transported in physical solution in blood of icefishes due to their loss of the circulating oxygen-binding protein hemoglobin (Hb) (Ruud 1954). As temperature increases, the oxygen content of icefish blood will decrease because oxygen solubility in water is inversely correlated with temperature. Moreover, the metabolic activity of tissues increases as temperature increases due to the Q_{10} effect, thereby elevating oxygen demand of the tissue. At the pejus temperature, oxygen supply will not match oxygen demand and tissues will become hypoxic (Portner 2001). Antarctic icefishes are more likely to experience a mismatch in oxygen supply and demand at lower temperatures than red-blooded species, potentially contributing to their lower thermal tolerance compared to red-blooded species.

Recent studies in our laboratory suggest that oxidative stress might also contribute to the lower thermal tolerance of icefishes compared to red-blooded species. Rates of reactive oxygen species (ROS) production increase *in vitro* in mitochondria of white- and red-blooded notothenioids as temperature increases (Mueller et al. 2011). This is likely more problematic for icefishes than red-blooded species for several reasons. First, icefishes have higher mitochondrial volume densities in their oxidative muscle than red-blooded notothenioids (O'Brien and Sidell 2000; O'Brien et al. 2003; Urschel and O'Brien 2008). High densities of mitochondrial membranes in icefishes may facilitate the production of ROS via the lipid peroxidation cycle (reviewed in Girotti 1998). Second, mRNA levels and enzyme activities of two important antioxidants, superoxide dismutase (SOD) and catalase are lower in oxidative muscles of Antarctic icefishes compared to red-blooded notothenioids, suggesting that icefishes are less protected against increases in ROS compared to red-blooded species (Cassini et al. 1993; Mueller et al. unpublished data). In addition, neither icefishes nor red-blooded species elevate their antioxidant

defenses in response to exposure to CTmax to counterbalance the temperature-induced increases in ROS production (Mueller et al. unpublished data), suggesting that, similar to the heat-shock protein response, Antarctic fishes might have lost their ability to up-regulate their antioxidant defense in response to warming (Carpenter and Hofmann 2000; Hofmann et al. 2000; Hofmann and Place 2005; Place et al. 2004).

Empirical evidence supports the hypothesis that icefishes are more sensitive to oxidative stress compared to red-blooded fishes as temperature increases. Levels of oxidized proteins and/or lipids significantly increased in heart ventricle of the icefishes *C. aceratus* and *C. rastrospinosus* in response to exposure to CTmax, but were unchanged in the red-blooded species *G. gibberifrons* and *N. coriiceps* (Mueller et al. unpublished data). We do not know however, how icefishes will respond to prolonged exposure to elevated temperature, which is likely to occur in their environment. The Western Antarctic Peninsula (WAP) region, in which water temperatures currently range between -1.8°C and +2°C throughout the year, is one of the fastest warming regions on Earth (Eastman 1993; Hofmann and Klinck 1998; IPCC 2001; Turner et al. 2005; Vaughan et al. 2003). Over the last 50 years, the WAP region experienced warming rates of near-surface air temperatures of 0.56°C per decade, which is 10-times faster than the global average (IPCC 2001; Turner et al. 2005; Vaughan et al. 2003). As a result, the summer temperatures of the upper water column have increased by more than 1°C over the last 50 years (Meredith and King 2005).

The aim of this study was to determine whether Antarctic notothenioids experience oxidative stress during prolonged exposure to elevated temperature. In addition, we sought to determine whether notothenioids up-regulate their antioxidant defense in response to prolonged exposure to warm temperature. The icefish *C. rastrospinosus* and the red-blooded notothenioid *N. coriiceps* were maintained at either at 0°C or 4°C for one week. Levels of oxidized proteins as well as transcript levels and

maximal activities of the antioxidants SOD and catalase were quantified in heart ventricle and pectoral adductor muscle.

3.3 MATERIAL AND METHODS

3.3.1 Animal and tissue collection

C. rastrospinosus (DeWitt and Hureau) and *N. coriiceps* (Richardson) were caught by otter trawl or baited traps, deployed from the ARSV *Laurence M. Gould* during the austral fall of 2009 in Dallmann Bay (64°08′S, 62°40′W), Antarctica. Animals were kept in circulating seawater tanks at 0 ± 0.5°C on board the *Laurence M. Gould* and then transferred to the US research station, Palmer Station, Antarctica. At the station, animals were held in circulating seawater tanks at 0 ± 0.5°C or 4 ± 0.5°C for one week. Animals were anesthetized in MS-222 (1:7500 in seawater) and killed by transection of the spinal cord. Tissues were quickly excised, frozen in liquid nitrogen and stored at -80°C until further use. All procedures were approved by the University of Alaska Fairbanks Institutional Animal Care Committee (134774-2).

3.3.2 Protein carbonylation

Levels of protein carbonylation were quantified in ventricle and pectoral adductor muscle of *C. rastrospinosus* and *N. coriiceps* held at 4°C. Levels of protein carbonyls in animals held at 0°C were quantified previously in our laboratory (Mueller et al. unpublished data). Protein carbonyls were measured based on the method by Levine and colleagues (Levine et al. 2000) using a Waters 1525 HPLC equipped with a Waters 2296 Photodiode Array Detector (Waters, Milford, MA, USA) and Zorbax 450 and Zorbax 250 gel filtration columns (Waters, Milford, MA, USA). Briefly, 10% tissue homogenates in 0.5 mol l^{-1} potassium phosphate buffer pH 7.8 were treated with a final concentration of at

least 1.2% streptomycin (10% stock solution made up in 50 mmol l^{-1} HEPES, pH 7.2) for 15 min at room temperature to precipitate nucleic acids, followed by centrifugation at 9,300 *g* for 10 min at 4°C. Proteins in the supernatant were precipitated with 1 ml of 100% ice-cold acetone for 30 min at -20°C and pelleted at 16,100 *g* for 15 min at 4°C. Protein pellets were washed with 1 ml of 80% ice-cold acetone and then resuspended in 100 μl of 6 mol l^{-1} guanidinium HCl, pH 2.5. Proteins were divided into four aliquots; two aliquots were incubated with 35 μl of 6 mol l^{-1} guanidinium HCl, pH 2.5 and two aliquots were incubated with 35 μl of 10 mM dinitrophenylhydrazine (DNPH) in 6 mol l^{-1} guanidinium HCl, pH 2.5 for 30 min at room temperature immediately prior to analysis with HPLC. Proteins were separated and eluted in 6 mol l^{-1} guanidinium HCl, pH 2.5 at a flow rate of 1 ml min^{-1}. Hydrazones, the reaction products of DNPH with protein carbonyls, were detected at 366 nm and normalized to the total protein content at 280 nm. Background levels of protein carbonyls were determined from the untreated protein aliquots and subtracted from protein carbonyl levels in the DNPH-treated aliquots as described by Levine and colleagues (Levine et al. 2000). All measurements were made in duplicate in 8 individuals per species. Levels of protein carbonyls were expressed as mmol carbonyls mol $protein^{-1}$.

3.3.3 Transcript levels of SOD1, SOD2 and CAT

Total RNA was isolated from ventricle of *C. rastrospinosus* and *N. coriiceps* held at 0°C or 4°C and transcribed into complementary DNA (cDNA) using RNeasy Fibrous Tissue Mini Kit (QIAGEN, Valencia, CA, USA) and TaqMan Reverse Transcriptase Reagents (Applied Biosystems, Carlsbad, CA, USA) as described by Orczewska et al (Orczewska et al. 2010). Gene-specific primer for amplifying SOD1, SOD2 and catalase (CAT) were designed using Primer Express v2.0 software (Applied Biosystems, Carlsbad, CA, USA), based on partial sequences, which were obtained previously in our laboratory (Appendices 2.2, 2.4, 2.6, 2.8, 2.10, 2.12, 2.14, 2.16), while gene-specific primers for 18S rRNA were previously published by Urschel and O'Brien (Urschel and O'Brien 2008)

(Table 3.1). Either the forward or the reverse primer of each primer set was designed over a splice site to ensure that genomic DNA was not amplified during quantitative real-time PCR (qRT-PCR) (Appendices 2.2, 2.4, 2.6, 2.8, 2.10, 2.12, 2.14, 2.16, 3.1, 3.2). Transcript levels of SOD1, SOD2, CAT and 18S rRNA were quantified by qRT-PCR using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) as described previously (Orczewska et al. 2010). Each qRT-PCR reaction contained 1X Power SYBR Green PCR Master Mix, 300 μM forward primer, 300 μM reverse primer and 5 ng cDNA (SOD1, SOD2, CAT) or 0.05 ng cDNA (18S rRNA). All measurements were made in triplicate in 7-8 individuals per species and temperature treatment. A standard curve of serially-diluted cDNA, pooled from both species and temperature treatments, was used to determine relative transcript levels. The relative transcript level of SOD1, SOD2 and CAT were normalized to transcript levels 18S rRNA, which was identified as a suitable housekeeping gene using BestKeeper v1 software as described previously by Orczewska et al. (Orczewska et al. 2010; Pfaffl et al. 2004).

3.3.4 Maximal activity of SOD (EC 1.15.1.1) and catalase (EC 1.11.1.6)

Maximal enzyme activity of SOD and catalase were quantified at 5 ± 0.5°C in ventricle and pectoral adductor muscle of *C. rastrospinosus* and *N. coriiceps* held at 4°C using a Perkin Elmer Lambda 25 spectrophotometer (Perkin-Elmer Corp., Waltham, MA, USA) equipped with a refrigerated, circulating water bath. Activity of SOD and catalase in animals held at 0°C was quantified previously in our laboratory (Mueller et al. unpublished data).

Activity of SOD was quantified by monitoring the reduction of cytochrome c at 550 nm (Crapo et al. 1978; McCord and Fridovich 1969). Briefly, tissues were homogenized in 9 volumes (v/w) of ice-cold 50 mmol l^{-1} potassium phosphate with 0.1 mmol l^{-1} EDTA, pH 7.8. The rate of reduction of 0.01 mmol l^{-1} acetylated cytochrome *c*

was determined in the absence of tissue homogenate in phosphate buffer containing 0.05 mmol l^{-1} xanthine, 0.01 mmol l^{-1} KCN and xanthine oxidase (XO). The final concentration of XO was determined empirically on a daily basis to obtain a rate of reduction of cytochrome *c* of 0.02 OD min^{-1}. One unit of SOD activity is defined as the amount of SOD needed to achieve 50% inhibition of the rate of reduction of cytochrome *c*. Tissue homogenate was diluted until a reduction rate of 0.01 ± 0.008 OD min^{-1} could be achieved. All measurements were made in triplicate in 4 individuals per species. Activity of SOD was expressed as Units g wet mass $tissue^{-1}$.

Activity of catalase was determined by following the breakdown of hydrogen peroxide at 240 nm as described by Beers and Sizer (Beers and Sizer 1952). Briefly, tissues were homogenized in 9 volumes (v/w) of 50 mmol l^{-1} phosphate buffer, pH 7.8. Rates of hydrogen peroxide decomposition were measured in 1 ml reaction volumes containing 50 mmol l^{-1} phosphate buffer, pH 7.8, 11 mmol l^{-1} hydrogen peroxide and 10 μl or 25 μl tissue homogenates. Hydrogen peroxide was replaced with 50 mmol l^{-1} phosphate buffer, pH 7.8 to measure background rates of hydrogen peroxide decomposition. All measurements were made in triplicate in 4-6 individuals per species. Activity of catalase was expressed as μmol min^{-1} g wet mass $tissue^{-1}$.

3.3.5 Statistical analysis

Significant differences in levels of oxidized proteins or antioxidants between different temperatures and tissues within a species, and between species at a common temperature were determined using a Student's *t*-test. All analysis were made using JMP7 (SAS, Cary, NC, USA) and significance was set at $P < 0.05$ unless otherwise noted. All data are expressed as mean ± s.e.m.

3.4 RESULTS

3.4.1 Levels of oxidized proteins

Levels of oxidized proteins did not increase in response to an increase in temperature in either heart ventricle or pectoral adductor muscle of either *C. rastrospinosus* or *N. coriiceps* ($P > 0.05$, Fig. 3.1 A, B). Levels of oxidized proteins were significantly lower in ventricles of the icefish compared to the red-blooded fish at 0°C and 4°C ($P < 0.05$, Fig. 3.1 A) but not significantly different between pectoral adductor muscles of either species at 0°C ($P > 0.05$, Fig. 3.1 B). However, carbonyl levels were significantly lower in pectoral adductor muscle of C. *rastrospinosus* compared to *N. coriiceps* at 4°C ($P < 0.05$, Fig. 3.1 B).

3.4.2 Transcript levels of antioxidants

Transcript levels of SOD1, SOD2 and CAT did not change in response to an increase in temperature in either *C. rastrospinosus* or *N. coriiceps* ($P > 0.05$, Fig. 3.2 A-C). mRNA levels of all antioxidants were similar in the heart ventricle between the two species at 0°C ($P > 0.05$, Fig. 3.2 A-C). At 4°C, transcript levels of SOD1 were similar between species ($P > 0.05$, Fig. 3.2 A), but levels of SOD2 and CAT were higher in *N. coriiceps* compared to *C. rastrospinosus* ($P < 0.05$, Fig. 3.2 B, C).

3.4.3 Maximal enzyme activity of antioxidants

Similar to transcript levels, maximal activity of SOD and catalase did not change in heart ventricle or pectoral adductor muscle in *C. rastrospinosus and N. coriiceps* in response to exposure to 4°C ($P > 0.05$, Fig 3.3 A-D). SOD and catalase activity were higher in heart ventricle and pectoral adductor muscle of the red-blooded fish compared to the icefish at 0°C and 4°C ($P < 0.05$, Fig 3.3 A-D).

3.5 DISCUSSION

The aim of this study was to determine whether Antarctic fishes experience oxidative stress and up-regulate their antioxidant defense in response to prolonged warming. Although rates of ROS production increased in isolated mitochondria of notothenioids in response to an increase in temperature (Mueller et al. 2011), neither levels of oxidatively damaged proteins, nor antioxidants increased *in vivo* in oxidative muscles of notothenioids following one week exposure to 4°C. Together these findings suggest that oxidative stress may not occur in Antarctic fishes in response to prolonged warming.

Oxidative stress might not have occurred in notothenioid fishes at 4°C for several reasons. First, exposing animals to 4°C for one week might not be sufficiently long enough to elicit a stress response. However, exposing the red-blooded *Trematomus bernacchii* to 4°C for just four hours resulted in an increase in more than 250 genes in the gills (Buckley and Somero 2009), suggesting that exposure of notothenioids to 4°C for one week should be sufficient to cause a stress response in these animals. Second, the Q_{10} coefficient for rates of mitochondrial ROS production might be similar or smaller than the Q_{10} for maximal activity of antioxidants in oxidative muscles of Antarctic notothenioids. Third, following one week at 4°C, animals may have become acclimated, resulting in a lower metabolic rate and lower rates of ROS production. Additionally, rates of ROS production may be reduced by alterations in membrane composition (Gille and Nohl 2001). Although membrane fluidity, which increases with temperature, may increase rates of ROS production (Gille and Nohl 2001), animals can attenuate fluidity-dependent increases in ROS production by reducing membrane fluidity through homeoviscous adaptation. Previous studies in fish suggest that both metabolic and membrane remodeling occur within one week of warm acclimation (Robinson and Davison 2008; Hazel and Landrey 1988). Whole animal oxygen consumption rates significantly declined between day 5 and day 9 of warm acclimation in *Pagothenia borchgrevinki*, and were equivalent to fish held at ambient temperature by day 9

(Robinson and Davison 2008). Additionally, in rainbow trout (*Oncorhynchus mykiss*) phospholipid head groups of renal membranes become modified within the first eight hours of acclimation to 20°C (Hazel and Landrey 1988). Further studies will be required to determine if notothenioids reduce their metabolic activity and/or ROS production within one week of exposure to 4°C.

The findings of this study are consistent with finding from our previous studies on the role of oxidative stress in Antarctic notothenioids (Mueller et al. unpublished data). Neither mRNA levels nor maximal enzyme activities of antioxidants increased in any tissue of white- or red-blooded species in response to exposure to CTmax (Mueller et al. unpublished data) or in this study in fishes held at 4°C for one week. Also similar to this study, levels of oxidized macromolecules did not increase in the heart ventricle of the red-blooded *N. coriiceps* in response to exposure to CTmax (Mueller et al. unpublished data). However, levels of oxidized lipids increased in the heart ventricle of the icefishes *C. aceratus* and *C. rastrospinosus* in response to exposure to CTmax, while levels of proteins only increased in heart ventricle of *C. aceratus* (Mueller et al. unpublished data). Levels of oxidized proteins might not increase in the icefish *C. rastrospinosus* in response to an elevation in temperature because its heart expresses the intracellular oxygen-binding myoglobin (Mb) (Moylan and Sidell 2000; Sidell et al. 1997; Vayda et al. 1997) and tends to have higher levels of antioxidants compared to icefishes that lack Mb such as *C. aceratus*. The presence of Mb in hearts of *C. rastrospinosus* might delay the onset of hypoxia as temperature increases compared to Mb-less icefishes. Hypoxia promotes the production of ROS because it disrupts the respiratory chain (Guzy and Schumacker 2006). Consequently, red-hearted icefishes might experience less severe increases in ROS as temperature increases compared to Mb-less icefishes. In addition, our previous studies suggests that the icefish *C. rastrospinosus* might be better protected against increases in ROS as temperatures increase compared to Mb-less icefishes. Maximal activity of catalase was 1.9-times higher in heart ventricle of *C. rastrospinosus* compared to *C. aceratus*, while maximal activity of SOD had the tendency to be higher in

the Mb-expressing icefish compared to the Mb-less icefish (student's *t*-test, $P = 0.0438$) (Mueller et al. unpublished data).

Additional quantification of levels of oxidized lipids in oxidative muscle of *C. rastrospinosus* and *N. coriiceps* after a one-week exposure to 4°C might further elucidate whether oxidative stress occurs in Antarctic fishes in response to prolonged increases in temperature.

3.6 ACKNOWLEDGMENTS

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3.7 FIGURES

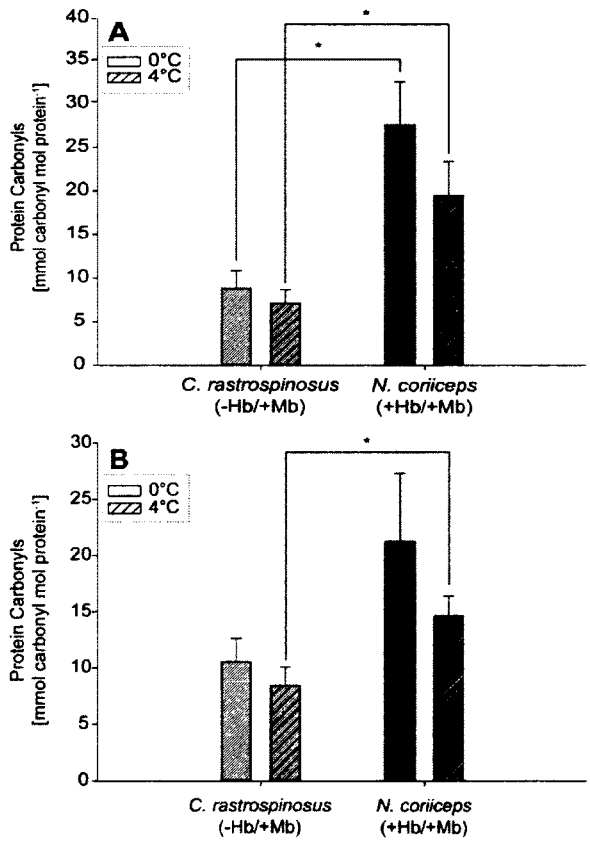

Figure 3.1 Level of oxidized proteins in Antarctic notothenioid fishes

Levels of oxidized proteins [mmol carbonyls mol protein^{-1}] in ventricle (A) and pectoral muscle (B) of *C. rastrospinosus* and *N. coriiceps*, held at 0°C (solid bars, Mueller et al. unpublished data) or 4°C (hatched bars) for one week. N = 7–11. Differences between species at a common temperature and significant differences between temperature treatments within a species are indicated by asterisks. $P < 0.05$.

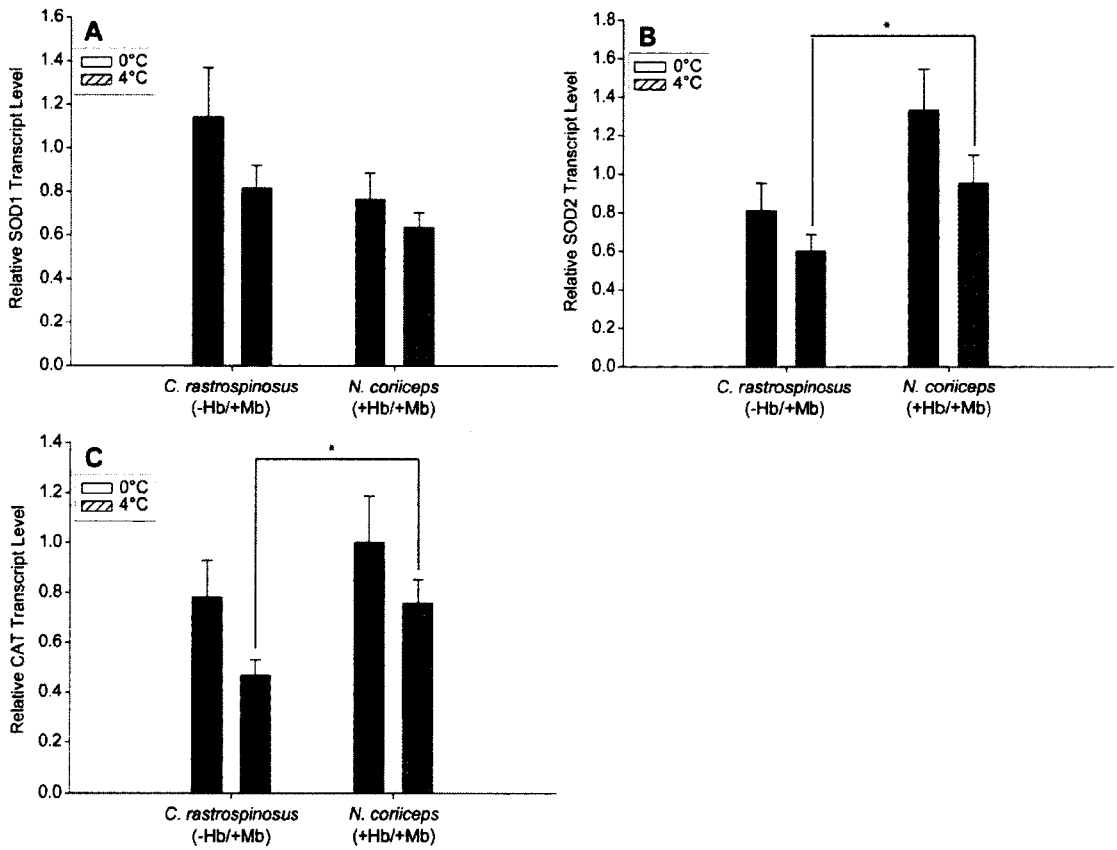

Figure 3.2 Antioxidant transcript levels in Antarctic notothenioid fishes

Relative transcript levels of SOD1 (A) and SOD2 (B) and CAT (C) in ventricle of *C. rastrospinosus* and *N. coriiceps*, held at 0°C (solid bars) or 4°C (hatched bars) for one week. Transcript levels of SOD1, SOD2 and CAT were normalized to transcript levels of 18S rRNA. N = 7–8. Differences between species at a common temperature and significant differences between temperature treatments within a species are indicated by asterisks. $P < 0.05$.

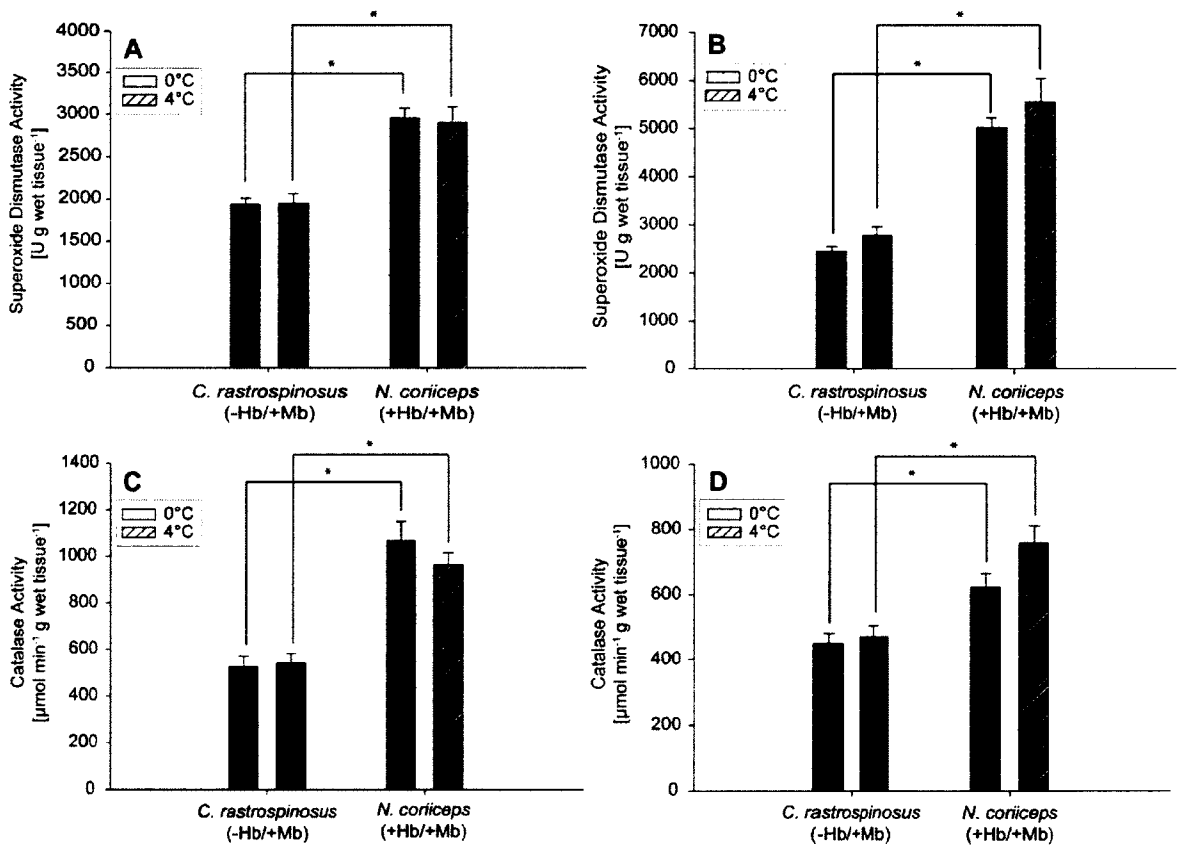

Figure 3.3 Antioxidant activities in Antarctic notothenioid fishes

Activity of SOD [Units g wet tissue^{-1}] (A, B) and CAT [μmol min^{-1} g wet tissue^{-1}] (C, D) in ventricle (A, C) and pectoral muscle (B, D) of *C. rastrospinosus* and *N. coriiceps*, held at 0°C (solid bars, Mueller et al. unpublished data) or 4°C (hatched bars) for one week. N = 4–8. Differences between species at a common temperature and significant differences between temperature treatments within a species are indicated by asterisks. $P < 0.05$.

3.8 TABLES

Table 3. 1 Gene-specific primers

Degenerate and gene-specific primers used for quantification of SOD1, SOD2, CAT and 18S rRNA transcript levels. Degenerate and gene-specific primers for 18S rRNA were previously published (Urschel and O'Brien, 2008).

Gene	Primer Type	Primer	Amplicon
SOD1	specific	F 5' CAAACGGGTGCATCAGTGC 3'	101 bp
		R 5' CACATTCCCCAGGTCTCCAA 3'	
SOD2	specific	F 5' GAGGAGAGCCACAGGGGG 3'	60 bp
		R 5' TCTGGAAGGAGCCAAAGTCCC 3'	
CAT	specific	F 5' CATGAAAGACCCCGACATGG 3'	82 bp
		R 5' GTCGCTGAACAAGAAAGACACCT 3'	
18S rRNA	specific	F 5' ACCACATCCAAGGAAGGCAG 3'	51 bp
		R 5' CCGAGTCGGGAGTGGGTAAT 3'	

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3.10 APPENDICES

ACTGTGGTAATCCTAGAGCTAATACATGCCAACGAACGCTGACCTTCGGGGA
TGCGTGCATTTATCAGACCCAAAACCCATGAGGGGTGCTCTCCGGGGCGCCC
CGGCCGCTTTGGTGACTCTAGATAACCTCGAGCCGATCGCTGGCCCTCGTGGC
GGCGACGTCTCATTCGAATGTCTGCCCTATCAACTTTCGATGGTACTTTCTGT
GCCTACCATGGTGACCACGGGTAACGGGGAATCAGGGATCGATTCCGGAGAG
GGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATT
TACCCACTCCCGACTCGGGGAGGTAGTGACGAAAAATAACAATACAGGACTC
TTTCGAGGCCCTGTAATCGGAATGAGCGCA

Appendix 3.1 Partial sequence of 18S rRNA in *C. rastrospinosus*

Partial sequence was previously published by Urschel and O'Brien 2008 (GenBank: EU857820). Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR.

ACTGTGGCAATCCCAGAGCTAATACATGCCAACGAACGCTGACCTTCGGGGA
TGCGTGCATTTATCAGACCCAAAACCCATGCGGGGTGCTCTCCGGGGTGCCC
CGGCCGCTTTGGTGACTCTAGATAACCTCGAGCCGATCGCTGGCCCTCGTGGC
GGCGACGTCTCATTCGAATGTCTGCCCTATCAACTTTCGATGGTACTTTCTGT
GCCTACCATGGTGACCACGGGTAACGGGGAATCAGGGTTCGATTCCGGAGAG
GGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAAT
TACCCACTCCCGACTCGGGGAGGTAGTGACGAAAAATAACAATACAGGACTC
TTTCGAGGCCCTGTAATCGGAATGAGTACA

Appendix 3.2 Partial sequence of 18S rRNA in *N. coriiceps*

Partial sequence was previously published by Urschel and O'Brien 2008 (GenBank: EU857819). Underlined regions indicate annealing sites of the specific forward and reverse primer used during qRT-PCR.

GENERAL CONCLUSION

Results from our studies suggest that icefishes might be more likely to experience oxidative stress than red-blooded species as temperature increases. Rates of production of reactive oxygen species (ROS) were similar between mitochondria from icefishes and red-blooded species at habitat temperature and increased in both as temperature increased. However, increases in ROS production might be more problematic for icefishes than red-blooded species. Icefishes have higher densities of mitochondrial membranes in their oxidative muscle compared to red-blooded species (O'Brien and Sidell, 2000; O'Brien et al., 2003; Urschel and O'Brien, 2008), and mitochondrial membranes, rich in polyunsaturated fatty acids promote the production of ROS via the lipid peroxidation cycle (reviewed in Girotti, 1998). In addition, icefishes are more likely to experience hypoxia as temperature increases, which also promotes the formation of ROS. Hypoxia interrupts the flow of electrons through the respiratory chain, promoting the production of ROS at complex III (Guzy and Schumacker, 2006). The effect of hypoxia on the respiratory chain can be mimicked in isolated mitochondria by the addition of antimycin A (Guzy and Schumacker, 2006). Mitochondria from icefishes had significantly higher rates of ROS production when inhibited with antimycin A than mitochondria of red-blooded species, most likely due to their higher mitochondrial membrane potential compared to red-blooded species. To compound the problem, mitochondria of icefishes are less protected against ROS. Total antioxidant capacity of icefish mitochondria per unit phospholipids is lower in icefishes compared to red-blooded notothenioids. Summarized, our studies on mitochondria isolated from heart ventricles of red- and white-blooded notothenioids suggest that icefishes might be more likely to experience oxidative stress in response to warming than red-blooded notothenioids.

These findings were confirmed by our study on oxidative stress in oxidative muscle of Antarctic fishes in response to exposure to their critical thermal maximum (CTmax). The CTmax is defined as the temperature at which animals lose equilibrium

and was determined by exposing animals to rapid, steady increases in temperature (3.6°C h^{-1}) until animals lost the ability to right themselves (Beers and Sidell, 2011). Levels of oxidized lipids increased in heart ventricle of icefishes *C. aceratus* and *C. rastrospinosus* but not red-blooded species *G. gibberifrons* and *N. coriiceps* in response to exposure to CTmax. In addition, levels of oxidized proteins also increased in heart ventricle of *C. aceratus,* but not in *C. rastrospinosus* or the two red-blooded species, in response to exposure to CTmax. Levels of oxidized macromolecules did not increase in pectoral adductor muscle of any species in response to exposure to CTmax. Interestingly, neither icefishes nor red-blooded species up-regulated their antioxidant defenses, and antioxidant levels tended to be lower in icefishes than red-blooded species.

Similar trends were found when the icefish *C. rastrospinosus* and the red-blooded *N. coriiceps* were exposed to prolonged increases in temperature. Neither levels of oxidized proteins nor levels of antioxidants increased in oxidative muscle of *C. rastrospinosus* and *N. coriiceps* in response to exposure to 4°C for one week. Levels of oxidized lipids, which increased in heart ventricle of *C. rastrospinosus* in response to exposure to CTmax, were not measured in this study. Future quantification of levels of oxidized lipids in oxidative muscle of white- and red-blooded notothenioids in response to exposure to 4°C will determine whether icefishes might be also more likely to experience oxidative stress in response to prolonged increases in temperature.

In summary, our studies on oxidative stress in Antarctic notothenioids as temperatures increases resulted in four key findings. First, icefishes are more sensitive to oxidative stress compared to red-blooded species as temperature increases, but this sensitivity differs between icefish species. The icefish *C. aceratus* might be more sensitive to oxidative stress than *C. rastrospinosus* for several reasons. Rates of ROS production were only quantified in mitochondria isolated from the heart ventricle of *C. aceratus* and *N. coriiceps*, so rates of ROS production might be lower *C. rastrospinosus* compared to *C. aceratus*. Furthermore, *C. aceratus* is one of the six species of icefishes

which does not express the intracellular oxygen-binding protein myoglobin (Mb) in their heart ventricle, while *C. rastrospinosus* does expresses the protein (Sidell et al., 1997; Vayda et al., 1997; Moylan and Sidell, 2000). Mb facilitates the diffusion of oxygen within the tissue and acts as oxygen reservoir, releasing oxygen under hypoxic conditions (reviewed in Sidell, 1998). The presence of Mb in the heart ventricle of *C. rastrospinosus* might delay the onset of hypoxia and minimize ROS production in these animals compared to *C. aceratus* as temperature increases. In addition, levels of catalase were higher in hearts of *C. rastrospinosus* compared to *C. aceratus* and levels of SOD also tended to be higher in hearts of *C. rastrospinosus* than *C. aceratus* (Student's *t* test, $P = 0.0438$). Together, this suggests that the red-hearted icefish might be better protected against increases in ROS as temperature increases than icefishes lacking Mb.

Second, our study indicates that although icefishes are more sensitive to oxidative stress as temperature increases, oxidative damage to cardiac proteins likely does not contribute to their lower thermal tolerance compared to red-blooded species. If the presence of oxidatively modified proteins reduces thermal tolerance of icefishes, then we would anticipate a lower CTmax for *C. aceratus* compared to *C. rastrospinosus*. Yet, the CTmax of the two icefish species is similar (Beers and Sidell, 2011). However, we cannot exclude the possibility that increases in levels of peroxidized lipids, which increased to the same extent in both icefish species, might contribute to their lower thermal tolerance. Even modest levels of membrane damage by ROS are associated with changes in the molecular organization of the lipid bilayer, which can affect the function of integral membrane proteins and disrupt membrane integrity (Mason et al., 1997).

Third, our studies revealed that sensitivity to oxidative stress in Antarctic icefishes is tissue specific. Hearts of icefishes experience oxidative stress in response to warming, whereas pectoral adductor muscles did not. These differences might be due to differences in rates of production of ROS, antioxidant levels and/or oxygen supply between the tissues. Rates of ROS production were only measured in mitochondria

isolated from the heart ventricle and not in mitochondria isolated from the pectoral adductor muscle. Rates of ROS production might be different between the two tissues due to differences in their metabolic activities. Maximal activity of cytochrome *c* oxidase per gram of tissue is up 1.3-times higher in heart ventricle of the two icefishes compared to their pectoral adductor muscle, suggesting that the heart ventricle has a higher maximal aerobic capacity (O'Brien and Sidell, 2000; O'Brien et al., 2003) and thus might produce ROS at higher rates. Maximal activity of SOD, the first antioxidant in the defense against ROS and the sole antioxidant detoxifying superoxide anions (reviewed in Chaudiere and Ferrari-Iliou, 1999), was higher in pectoral adductor muscle compared to heart ventricle, suggesting that the latter might be less protected against ROS. Moreover, hearts of icefishes are more likely to experience hypoxia than pectoral adductor muscles. While hearts are supplied with oxygen from venous blood entering the ventricular lumen, the pectoral muscle is supplied with freshly-oxygenated blood via a hypobranchial shunt (Agnisola and Tota, 1994; Zummo et al., 1995; Egginton and Rankin, 1998).

Fourth, our results suggest that Antarctic notothenioids have lost the ability to up-regulate their antioxidant defenses in response to warming. Antarctic notothenioids are well adapted to their constantly cold environment. Their high degree of stenothermy is concomitant with the loss of some thermal plasticity. For example, while Antarctic notothenioids express basal levels of heat shock proteins, they do not up-regulate expression when heat shocked, which is a highly conserved stress response found in species from all taxa (Feder and Hofmann, 1999; Carpenter and Hofmann, 2000; Hofmann et al., 2000; Place et al., 2004; Hofmann and Place, 2005).

Future studies should focus on identifying the physiological basis of lower thermal tolerance in icefishes compared to red-blooded fishes. A recent study by Beers and Sidell showed that the CTmax of Antarctic notothenioid fishes correlates with circulating levels of Hb in these animals (Beers and Sidell, 2011). This suggests that the thermal tolerance of icefishes is reduced due the low oxygen-carrying capacity of their

blood and/or adaptations associated with the loss of Hb. The thermal tolerance of an organism can be defined by it's ability to match oxygen supply and oxygen demand over a range of temperatures (Portner, 2001). The low oxygen-carrying capacity of icefish blood might result in a mismatch of oxygen supply and demand more quickly during warming compared to red-blooded species. Our studies suggest that mitochondrial adaptations, such as increased mitochondrial volume densities and altered morphology, make heart of icefishes more sensitive to oxidative stress as temperatures increase. Further studies are needed to address whether the increase in peroxidized lipids in icefishes in response to warming might impair cardiac function and contribute to their lower thermal tolerance. Future studies should also address whether differences in the ability of icefishes and red-blooded species to maintain cardiac or neuronal function might also contribute to differences in thermal tolerance.

Future studies might also investigate the transcriptional regulation of antioxidants in notothenioid fishes. Gene regulatory regions and transcription factors could be compared between Antarctic notothenioids and the closely-related New Zealand notothenioid, *Notothenia angustata* to determine whether changes in DNA-binding sites and/or their regulatory binding sites might prevent an up-regulation of antioxidants in Antarctic notothenioids in response to warming. It is unknown whether *N. angustata*, which inhabits warmer waters, has maintained this capacity.

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